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# ditorial

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# Palmitic Acid Transport in Platelets of Normal Subjects and of Patients with Liver Cirrhosis

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**Key Words.** Liver cirrhosis Palmitic acid Platelets Transport

**Abstract.** Bovine serum albumin bound  $^{14}\text{C}$ -palmitic acid (BSA- $^{14}\text{C}$  PA) is transported into platelets of normal and cirrhotic subjects by simple diffusion. Initial uptake increases linearly with the concentration of BSA- $^{14}\text{C}$  PA in the medium. The time course accumulation of BSA  $^{14}\text{C}$  PA is found to be higher in the platelets of patients with cirrhosis compared to that of normals. At 50-min incubation, the amount of  $^{14}\text{C}$  PA accumulated in the platelets of cirrhotic subjects is  $52.43 \pm 7.40$  nmol/ $10^8$  platelets and in the platelets of normal controls it is  $22.71 \pm 3.14$  nmol/ $10^8$  platelets. The diffusion rate of BSA- $^{14}\text{C}$ -PA is also higher in the platelets of cirrhotic patients where the slope of the concentration dependency curve for  $10^8$  platelets at 30 sec is  $56.0 \pm 4.8 \times 10^{-4}$  liters. This value is  $21.6 \pm 2.4 \times 10^{-4}$  liters for normal subjects.

## Introduction

The albumin bound free fatty acid uptake by platelets is established through simple diffusion and is dependent on the free fatty acid to albumin ratio on the chain length of the fatty acid and on the degree of saturation (15). Between the fatty acids, saturated and monounsaturated fatty acids are preferentially taken up by platelets from the surrounding (15). Both the free fatty acid and

albumin is dissociated during uptake (15). Newly incorporated free fatty acids are located at the membrane surface (11) and in short term incubations they remain in unesterified form (15). As the incubation continues increasing amounts of free fatty acids are oxidized to  $\text{CO}_2$  and incorporated into platelet lipids. Each fatty acid has a distinctive pattern of incorporation into different lipid fractions of platelets (6, 7). After 60 min incubation 35% of  $^{14}\text{C}$  palmitic acid is converted to  $\text{CO}_2$ , 36% to phospholipids, 4% to glycerides and 25% remain as palmitic acid (PA) (15). The distribution of PA formed intracellularly by *de novo* syn-

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thesis is the same as with the distribution of PA taken up from the medium (8).

PA, like other long chain, saturated fatty acids, plays an important role in thrombus formation and in aggregation and adhesion of platelets (10, 12, 13). In patients with liver cirrhosis, together with thrombopenia and defective platelet adhesion and aggregation (3), the platelet total lipid (9) and total phospholipid contents of platelets are decreased (1). Increased fibrinolytic activity in these cases has also been reported (3).

In the present investigation, the PA transport in the isolated platelets of cirrhotic subjects having decreased serum cholesterol, serum total lipid, platelet total phospholipid contents and defective platelet aggregation is studied in comparison with the PA transport in isolated platelets of normal subjects.

### Materials and Methods

Venous blood was taken with 0.077 M disodium ethylenediaminetetraacetate (EDTA), pH 7.4, with ratio of 9:1, from normal donors over aging 50 years of age and from patients with portal liver cirrhosis with defective platelet aggrega-

tion (4) and with decreased levels of serum cholesterol (2), serum total lipid (10) and platelet total phospholipids (14) (table I). A platelet pellet was obtained from platelet-rich plasma (PRP) by centrifugation at 750 g for 20 min and was washed once with Tris-NaCl buffer (0.03 M Tris-HCl + 0.12 M NaCl + 0.003 M EDTA, pH 7.4). Washed platelets were suspended in the same buffer without EDTA to give final platelet count of  $1.3 \times 10^8$  platelets/ml (5). In all experiments the transport assays were done within 2 h after the blood was drawn from the donors. 1 patient and 1 control were tested at the same time.

Preparation of bovine serum albumin (BSA) bound 1-<sup>14</sup>C-palmitic acid (BSA-<sup>14</sup>C-PA) PA (Nutritional Biochemical Corp.) was dissolved in 1 ml of benzene to give a final concentration of 2.2 mM. 10  $\mu$ Ci <sup>14</sup>C-PA (Radiochemical Centre, Amersham, England specific activity 57.9 mCi/mmol, 99% purity) was added. After evaporating benzene in a rotary evaporator 1 ml of Tris-HCl buffer containing 0.5% BSA (Behringwerke AG) was added. This solution was mixed for 30 min; the remaining benzene was evaporated and albumin-bound <sup>14</sup>C-PA solution was kept at 20 °C until use.

**Transport Assay** For time course accumulation studies, upon preincubation of platelet suspension at 37 °C (Gruet water bath and shaker) and at 4 °C (ice bath) for 15 min, the transport assay was initiated with the addition of BSA-<sup>14</sup>C-PA. In most experiments the final concentration of BSA-<sup>14</sup>C-PA was  $2.2 \times 10^{-6}$  M (specific activity

Table I. Serum cholesterol, serum total lipid, platelet total phospholipid and ADP-induced aggregation at 0.5  $\mu$ g/ml ADP concentration values of normal and cirrhotic subjects (mean  $\pm$  SD)

	Cirrhotic		Normal
Cholesterol, mg% (n = 10)	162.5 $\pm$ 14.2		221.0 $\pm$ 22.7
		p < 0.05	
Total lipid, mg% (n = 10)	625.6 $\pm$ 47.4		834.4 $\pm$ 34.3
		p < 0.01	
Platelet total phospholipids, $\mu$ g PL/ $10^8$ platelets (n = 12)	7.63 $\pm$ 1.29		10.88 $\pm$ 0.83
		p < 0.001	
ADP-induced aggregation II wave max. amp., cm (n = 10)	3.02 $\pm$ 0.76		5.94 $\pm$ 0.94
		p < 0.05	



ity: 4.56 mCi/nmol). At certain time intervals 0.5-ml aliquots were withdrawn and passed through Millipore filters (HAWP 02500 filters, Millipore Corp.) with the use of a Millipore manifold. The filters were washed 3 times with 2 ml of cold Tris-NaCl albumin solution (0.03 M Tris-HCl + 0.12 M NaCl, 0.5% BSA). The washing was done in 30 sec. The Millipore filters were transferred into scintillation vials and were dried at 37 °C overnight. Toluene-spectrofluor (Amersham/Searle) was added as cocktail and the radioactivity was counted in a Nuclear-Chicago Mark II liquid scintillation counter. Quenching corrections were made for each sample. The results were expressed as nanomoles of PA taken up per  $10^6$  platelets.

**Kinetic Studies.** 1 ml of platelet suspension was incubated in different concentrations of BSA- $^{14}$ C PA for 30 sec both at 37 and 4 °C, where the albumin to  $^{14}$ C PA ratio was constant. The transport reaction was terminated by the addition of 5 ml of cold washing solution (Tris-NaCl-albumin) and passing through Millipore membranes immediately. Membranes were washed 3 times with washing solution and radioactivity was determined in the same way as in the transport assay. The results were expressed as nanomoles of PA taken up per  $10^6$  platelets per 30 sec.

## Results

The time course accumulation of BSA- $^{14}$ C PA by platelets of normal and cirrhotic subjects at 37 and 4 °C is shown in figure 1.  $^{14}$ C PA is rapidly taken up by the platelets from the incubation medium. As the time progresses the radioactivity in the platelets decreases approaching a constant value at about 1 h. No difference in initial uptake is observed at 37 and 4 °C (fig. 2) but the amount of  $^{14}$ C PA accumulated at the equilibrium stage is higher at 4 °C (fig. 1). The same results are observed with platelets of cirrhotic patients (fig. 1 2).

The averages of  $^{14}$ C PA uptake by platelets up to 100 min for 8 cirrhotic and 8 normal subjects are presented in table II.

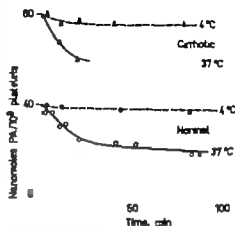


Fig. 1. Time course accumulation of BSA- $^{14}$ C PA by platelets of normal and cirrhotic subjects at 37 and 4 °C. The uptake is initiated with the addition of BSA  $^{14}$ C PA to give a final concentration of  $2.2 \times 10^{-6}$  M. At certain time intervals aliquots are Millipore filtered and radioactivity is determined. This is an example representative of several other experiments. O = Normal at 37 °C; ● = normal at 4 °C; Δ = cirrhotic at 37 °C; ▲ = cirrhotic at 4 °C.

Both the initial  $^{14}$ C PA uptake and the amount accumulated in later times are found to be statistically higher about 2–2.5 times, in platelets of patients with cirrhosis than of normal controls. There is a linear relationship between the initial  $^{14}$ C PA uptake by platelets and the  $^{14}$ C PA concentration in the incubation medium as seen in figure 2. The initial  $^{14}$ C-PA uptake by platelets is the same at 4 and 37 °C, both in normals and in cirrhotic patients. There is 2.6 times more initial uptake by platelets of patients with cirrhosis than that of normal controls (fig. 2) and the slopes of the curves obtained from the plot of initial  $^{14}$ C PA uptake at 30 sec by  $10^6$  platelets vs.  $^{14}$ C PA concentration in the external medium are  $21.6 \pm 2.4 \times 10^{-4}$  liters for normal controls (mean  $\pm$  SE,  $n = 11$ ) and  $56.0 \pm 4.8 \times 10^{-4}$  liters for cirrhotic patients (mean

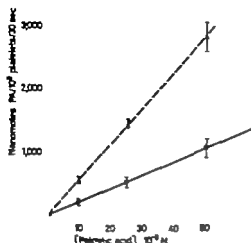


Fig. 2. Concentration dependency curves at BSA- $^{14}\text{C}$ -PA transport in platelets of normal and cirrhotic subjects. Initial uptakes are determined by incubating the platelets for 30 sec at different concentrations of BSA- $^{14}\text{C}$ -PA up to  $5.0 \times 10^{-4} \text{ M}$  in the medium. The reaction is terminated with the addition of cold Tris-NaCl-albumin solution and Millipore filtered immediately. The points and bars represent mean  $\pm$  SE of 11 normal and 11 cirrhotic subjects. O = Normal at  $37^\circ\text{C}$  and  $4^\circ\text{C}$ ,  $\Delta$  = cirrhotic at  $37$  and  $4^\circ\text{C}$ .

$\pm$  SE,  $n = 11$ ). The difference is statistically significant.

## Discussion

The initial uptake of  $^{14}\text{C}$  PA both by isolated platelets of cirrhotic and normal subjects is energy and temperature independent being the same at  $37$  and  $4^\circ\text{C}$ . The nanomoles of  $^{14}\text{C}$  PA taken up per  $10^9$  platelets per 30 sec increases linearly with BSA- $^{14}\text{C}$  PA concentration in the incubation medium. No saturation kinetics are observed in PA concentrations up to  $5 \times 10^{-4} \text{ M}$ . Therefore, PA is transported

Table II. Accumulation of  $^{14}\text{C}$ -PA in the isolated platelets of normal and cirrhotic subjects at  $37^\circ\text{C}$

Incubation time min	Normal ( $n = 8$ ) nanomoles PA/ $10^9$ platelets (mean $\pm$ SE)	Cirrhotic ( $n = 8$ ) nanomoles PA/ $10^9$ platelets (mean $\pm$ SE)
1	$39.45 \pm 3.33$	$75.57 \pm 15.63$
5	$13.00 \pm 3.47$	$69.00 \pm 10.03$
10	$28.71 \pm 3.50$	$64.57 \pm 9.05$
20	$25.29 \pm 3.04$	$61.29 \pm 10.13$
30	$22.71 \pm 3.14$	$52.43 \pm 7.40$
100	$22.43 \pm 3.14$	$51.57 \pm 7.66$

$p < 0.05$

$p < 0.01$

$p < 0.01$

$p < 0.01$

$p < 0.01$

$p < 0.01$

into the platelets of both cirrhotic and normal subjects by simple diffusion. Our results are in accordance with the literature findings on normals (15).

BSA-bound  $^{14}\text{C}$  PA is incorporated into platelets immediately but the amount accumulated is decreased when time progresses reaching a constant value. The CPA which is washed away by PA-free Tris-NaCl-albumin solution is the PA loosely bound to membrane. The PA which cannot be washed away by Tris-NaCl-albumin solution is in the metabolic pool which is metabolized to CO and is incorporated into phospholipids and into glycerides. Therefore, the increase observed in the accumulation of  $^{14}\text{C}$  PA at later times at  $4^\circ\text{C}$  is due to decreased levels of metabolic activity at this temperature.

In the present work the BSA-bound  $^{14}\text{C}$  PA transport, both the diffusion rate and the amount accumulated in later times

of incubation, is increased significantly in the platelets of cirrhotic subjects compared to normals. All the cirrhotic subjects used in these experiments had low levels of cholesterol and total lipid in their serum, at the same time they had decreased content of total phospholipid in their platelets. Therefore increased diffusion rate of PA and increased accumulation of PA in the platelets of cirrhotic subjects is due to the starvation of platelets from PA.

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## NADH Cytochrome b<sub>5</sub> Reductase in Platelets and Leukocytes with Special Reference to Normal Levels and to Levels in Carriers of Hereditary Methemoglobinemia with or without Neurological Symptoms

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**Key Words.** Hereditary methemoglobinemia Leukocyte  
NADH-cytochrome b<sub>5</sub> reductase Platelet

**Abstract.** Normal levels of NADH-cytochrome b<sub>5</sub> reductase activity in platelets, lymphocytes and granulocytes were determined. The homogenate of each cell was treated with Triton X 100 after incubation with lipoprotein lipase. The reductase was extracted very well from the cells by this treatment. Moreover the assay of the reductase activity in the cells became accurate and reproducible by the treatment. The reductase level of each cell was also determined in cases of hereditary methemoglobinemia. It was normal in the case of the disease without mental retardation, and low with mental retardation. This latter case might be due to the deficiency of cytochrome b<sub>5</sub> reductase in the whole tissue.

NADH-cytochrome b<sub>5</sub> reductase is the major enzyme catalyzing the reduction of methemoglobin *in vivo*, and hereditary methemoglobinemia is now considered to be due to a deficiency of this enzyme [1-2]. Recently Leroux *et al.* [3] described two types of enzyme deficiency by measuring NADH-cytochrome b<sub>5</sub> reductase activity in leukocytes and other tissues (so-called erythrocyte type and generalized type). In most cases of hereditary methemoglobinemia the enzyme defect is restricted to the erythrocytes and the patients exhibit slate

gray cyanosis but encounter little difficulty in daily work (erythrocyte type). However it has been evident that a small but significant number have mental retardation [4-6]. In these cases, the cyanosis is associated with a progressive encephalopathy and the enzyme defect is also found in leukocytes and other tissues (generalized type).

In this report, an improved method for the determination of cytochrome b<sub>5</sub> reductase activity in platelets and leukocytes is developed in order to obtain precise normal levels. This method is also applied to deter

mination of the enzyme activity in platelets and leukocytes from some patients with hereditary methemoglobinemia.

## Materials and Methods

### Blood Samples

Normal human blood samples were obtained from healthy students and officials who were invited to volunteer at our University. The blood samples of patients with hereditary methemoglobinemia were kindly offered by Dr. Hirano of Fujita-Gakuen University Hospital for the erythrocyte-type enzyme deficiency [7], and by Dr. Hara of Yokohama Keiyuh Hospital for the generalized-type enzyme deficiency [8]. The samples were collected by plastic syringe from the antecubital vein and poured into plastic equipment containing EDTA  $K_2$  (2.7 mg/ml of blood) as anticoagulant.

### White Blood Cell Collection

All samples were handled in plastic equipment. The blood was centrifuged at 120 g for 10 min at room temperature and the supernatant platelet-rich plasma was removed by aspiration. From this supernatant the platelet specimens were collected. Leukocyte collection was performed according to the modified method of Bøyum [9]. The bottom fraction containing erythrocytes and leukocytes was washed once with saline and centrifuged again at 120 g for 10 min. To the washed fraction an equal volume of 2 g/dl dextran (D-500, Sigma Chemical, St. Louis, Mo.) in saline was added and allowed to stand for 15 min at room temperature. After sedimentation of erythrocytes, leukocytes in the supernatant fraction were collected by centrifugation at 600 g for 10 min and washed twice with saline. The washed cells were suspended in about 2 ml of saline. The suspension was layered on top of 3 ml metrizoate Ficoll solution which contained 29.4 parts of 1,200 g/ml density solution of sodium metrizoate (Nyegaard, Oslo) and 70.6 parts of 8 g/dl solution of Ficoll-400 (Pharmacia Fine Chemicals, Uppsala), and centrifuged at 400 g, 4°C for 30 min. Lymphocytes at the interface and granulocytes in the bottom fraction were collected by centrifugation at 600 g for 10 min. Erythrocytes remaining in the granulocyte fraction were subsequently hemolyzed with 0.28 g/

dl NaCl solution. These cells were washed once with saline and suspended in the same medium.

The purity of the platelet, lymphocyte or granulocyte fraction was tested by microscopic observation. The platelet and lymphocyte fractions have almost 100% purity and the yield of each cell was 40–50 and 30–32%, respectively. The granulocyte fraction has 82–94% purity and its remaining parts were lymphocytes, and the yield of granulocytes was 42–58%. These yields were obtained when the separation was carried out within 48 h after collecting blood.

There is no significant decline in diaphorase or cytochrome  $b_5$  reductase activity after platelets or leukocytes were stored at 4°C for 5 days and erythrocytes were stored at 4°C for 25 days [10].

### Homogenization and Extraction of the Enzyme

Platelet and leukocyte suspension were homogenized for 2 min in a Teflon homogenizer. Extraction of the enzyme was performed at pH 7.4 in a 100-mmol/l Tris-HCl buffer containing the homogenates with 100–300 mg of protein and several kinds of detergents with a final concentration giving maximum specific activity of the enzyme under such conditions. Protein concentration was determined by the method of Lowry *et al.* [11] using bovine serum albumin as a standard.

### Enzyme Assay

NADH-diaphorase activity was assayed by a method based on that of Scott [12] as previously described [10]. The assay mixture for the enzyme activity contained 50  $\mu$ mol Tris-HCl buffer pH 7.4, 0.2  $\mu$ mol 2,6-dichlorophenolindophenol, 1  $\mu$ mol EDTA  $Na_2$ , 0.4  $\mu$ mol NADH, and 1.0 ml enzyme space in a total volume of 1.5 ml. The reaction was started by addition of NADH and the initial rate of reduction of the dye at 600 nm was recorded with a Hitachi model 200 recording spectrophotometer at 25°C. The enzyme activity was expressed as nanomole of electron acceptor per minute per milligram of protein for platelets and leukocytes, and as nanomole of electron acceptor reduced per minute per milligram of hemoglobin for erythrocytes. The millimolar extinction coefficient of 20.1 for the oxidized dye was used for the calculation of the enzyme activity.

NADH-cytochrome  $b_5$  reductase activity was assayed by the method as described previously [10]. The assay mixture contained 20  $\mu$ mol phos-

phate buffer, pH 7.0, 1  $\mu$ mol EDTA Na<sub>2</sub>, 0.004  $\mu$ mol cytochrome b<sub>5</sub> purified from rabbit liver [13], 0.4  $\mu$ mol NADH, and enzyme in a total volume of 1.5 ml. The reaction procedure was the same as that in the diaphorase assay. The millimolar extinction coefficient of 100 at 424 nm was used for the difference between the oxidized and reduced form of cytochrome b<sub>5</sub>.

## Results

### Variation in Enzyme Activity of the Separated Cells

The NADH-methemoglobin reductase activity of platelets, lymphocytes, and granulocytes was measured as diaphorase using cell homogenates from various blood sam-

ples as shown in table I. It was observed that diaphorase activity of the homogenates became gradually higher during the period of standing after homogenization of the cells at 4°C. Furthermore, large variations of diaphorase activities were observed as shown in the last line of table I.

### Effect of Detergent on Enzyme Extraction

In order to obtain a constant level of the diaphorase activity of the cells, the enzyme was extracted with several kinds of detergent under the conditions described in Materials and Methods. As shown in table II, treatment of the homogenate with Triton X 100 for 15 min after incubation with li-

Table I. Variation in NADH-methemoglobin reductase (diaphorase) activity of platelets, lymphocytes and granulocytes separated from normal human blood

Hours standing at 4°C	Platelet homogenate	Lymphocyte homogenate	Granulocyte homogenate
0	10.0	5.1	1.1
3	11.4	6.3	1.3
6	11.2	6.6	
10		6.5	2.5
18	11.6	6.6	2.7
24	13.9	8.2	2.9
48	19.7	8.4	2.9
Mean $\pm$ 2 SD after standing at 4°C for 24 h (sample size)	22.3 $\pm$ 17.8 (n = 17)	8.2 $\pm$ 3.3 (n = 20)	2.2 $\pm$ 1.6 (n = 9)

Diaphorase activity in the homogenate of each cell type was measured ( $\mu$ mol/min/mg of protein).  
— = Undetermined.

Table II. Effect of detergents on NADH-methemoglobin reductase (diaphorase) activity of platelets and leukocytes separated from normal human blood

Detergent	Final concentration	Platelet	Lymphocyte
Sodium dodecylsulfate	0.1 mmol/l	10.6	4.6
Deoxycholate-Na	1.0 mmol/l	22.2	5.0
Lipoprotein lipase (LPL)	1.50 units/ml	4.9	0.9
Triton X-100	1.0 mg/ml	28.6	11.8
LPL + deoxycholate		20.2	8.8
LPL + Triton X-100		42.6	14.9

The homogenate of each cell type was incubated with LPL for 30 min and with other detergents for 15 min, respectively under the condition described in the text. — The homogenate was incubated with LPL (150 units/ml) and subsequently treated with deoxycholate (1.0 mmol/l) or Triton X-100 (1 mg/ml). After the incubation, it was centrifuged for 10 min at 600 g and diaphorase activity ( $\mu$ mol/min/mg of protein) of the supernatant was measured.

Table III. NADH methemoglobin reductase activities in extracts of platelets, leukocytes and erythrocytes separated from normal human blood or methemoglobinemic patients' blood

Cell sources	Diaphorase activity			Cytochrome b <sub>5</sub> reductase activity		
	normal mean $\pm$ 2 SD (sample size)	hereditary methemo- globinemia		normal mean $\pm$ 2 SD (sample size)	hereditary methemo- globinemia	
		erythrocyte type (Toyoake)	generalized type (Yokohama)		erythrocyte type (Toyoake)	generalized type (Yokohama)
Platelet	40.1 $\pm$ 13.9 (n = 20)	41.1	3.3	20.6 $\pm$ 7.4 (n = 15)	18.8	2.2
Lymphocyte	14.5 $\pm$ 8.0 (n = 12)	12.8	2.1	6.0 $\pm$ 2.9 (n = 9)	5.9	< 0.01
Granulocyte	5.6 $\pm$ 1.6 (n = 13)	4.5	< 0.01	2.6 $\pm$ 0.9 (n = 7)	2.1	< 0.01
Erythrocyte	0.40 $\pm$ 0.16 (n = 17)	0.03	< 0.01	0.39 $\pm$ 0.08 (n = 19)	< 0.01	< 0.01

Procedure for extraction of the enzyme with LPL + Triton X 100 from platelets or leukocytes is the same as that in table II. Enzyme activity nmol/min/mg of protein for platelets and leukocytes, and nmol/min/mg of hemoglobin for erythrocytes.

poprotein lipase for 30 min at room temperature (LPL + Triton X 100 method) gave the highest activity of diaphorase compared with five other detergent treatments.

#### *Levels of Enzyme Activities of the Separated Cells*

NADH methemoglobin reductase levels of platelets and leukocytes from numerous samples of normal human blood were determined by using the extraction method with detergents (LPL + Triton X 100 method). Table III shows the levels of diaphorase activities in platelets, lymphocytes and granulocytes and the levels of cytochrome b<sub>5</sub> reductase which plays a major role in reducing methemoglobin *in vivo*.

The reductase levels in patients with he-

reditary methemoglobinemia are also shown in table III. In 1 patient, Toyoake (erythrocyte type) both diaphorase and cytochrome b<sub>5</sub> reductase levels in all cells were within a normal range, while in another patient, Yokohama (generalized type) significantly low reductase levels were observed in each cell. In erythrocytes from these patients both diaphorase and cytochrome b<sub>5</sub> reductase activities were almost negligible.

#### **Discussion**

*Leroux et al.* [3] postulated that hereditary methemoglobinemia with neurological involvement might result from a generalized enzyme defect, and demonstrated a lack of

NADH-diaphorase and cytochrome  $b_5$  reductase not only in erythrocytes but also in several tissues, leukocytes, muscle, liver and fibroblasts from a patient with this type of methemoglobinemia.

Of these several tissues, leukocytes as well as erythrocytes are easier to obtain from patients. Therefore the use of leukocytes for measuring the enzyme activity appears to be satisfactory for distinguishing the generalized-type enzyme defect from the erythrocyte-type defect. It is evident from the present study however that diaphorase activity levels in platelets, lymphocytes and granulocytes are themselves not identical. The diaphorase activity level in each cell, moreover is variable in relation to standing period after separation of the cells. The observed changes in cytochrome  $b_5$  reductase activity in these cells may be attributed to the breakdown of a complex with the reductase and lipoprotein.

The present LPL + Triton X 100 method for extraction of the enzyme gave a reproducible value of diaphorase and cytochrome  $b_5$  reductase activity for each cell when repeated measurements by this method were performed on several normal blood samples. The values for diaphorase activity are greater than those for cytochrome  $b_5$  reductase activity as shown in table III. This is probably due partly to the difference in millimolar extinction coefficient of the substrate electron acceptor and partly to the characteristics of diaphorase assay. It was shown by ion-exchange chromatographic study that diaphorase present in normal human erythrocytes is resolved into various fractions [14, 15] and the conventional diaphorase assay using the dye 2,6-dichlorophenolindophenol as electron acceptor quantifies cytochrome  $b_5$  reductase together

with parallel quantification of those enzyme known to act like diaphorase [16].

Regarding the hereditary methemoglobinemic samples of platelets and leukocytes, the present LPL + Triton X 100 method showed a clear-cut distinction between samples with generalized-type enzyme defect and those with erythrocyte-type enzyme defect or normal samples. Several other enzymes chosen as internal standards were also measured, glucose-6-phosphatase and lactate dehydrogenase activities for platelets and lymphocytes from a patient with the generalized-type enzyme defect were all in the normal range. Although we have seen no report determining cytochrome  $b_5$  reductase activity with special reference to platelets, platelets may be recommended for such a distinction of the types because of their higher levels of cytochrome  $b_5$  reductase activity in normal samples. The diaphorase activities in platelets and lymphocytes from one heterozygote (erythrocyte type) were 51.5 and 18.8 nmol/min/mg of protein, and cytochrome  $b_5$  reductase activities in these cells were 22.1 and 8.3 nmol/min/mg of protein, respectively.

The reason why mental retardation or neurological disorder are associated with the cytochrome  $b_5$  reductase defect in platelets and leukocytes is obscure. Precise determination of cytochrome  $b_5$  reductase activity in platelets and leukocytes is important for diagnosis of the generalized-type enzyme defect in hereditary methemoglobinemia with these symptoms. Our method may find practical use for this purpose.

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## Defect of Bone Marrow Granulocyte Reserve in Rheumatic Diseases Evaluated with Etiocholanolone

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**Key Words.** Bone marrow · Etiocholanolone · Granulocyte reserve · Rheumatoid arthritis

**Abstract.** In 10 patients with rheumatoid arthritis, with normal baseline granulocyte count, the etiocholanolone test showed a decrease in the bone marrow granulocyte reserve; factors causing such a defect are discussed.

### Introduction

The bone marrow granulocyte reserve can be studied by the response of peripheral granulocytes to injection of pyrogens [Mechanic and Frey 1961, Fink and Calabresi 1962], etiocholanolone [Vogel *et al.* 1967, Karjalainen and Wasanjeru 1973] and cortisone [Dale *et al.* 1975]. These tests are very useful when peripheral granulocytopenia is still absent or about to occur.

Since leukopenia is frequently found in patients with rheumatic diseases such as rheumatoid arthritis, systemic lupus erythematosus, Felty's syndrome, psoriatic arthritis, Sharp's syndrome, we have used the etiocholanolone test to investigate the bone marrow reserve in a group of subjects with various types of rheumatic disease, and we have found a significant decrease in the peripheral granulocyte response to the injection of etiocholanolone in subjects with rheumatoid arthritis.

### Subjects and Methods

#### Subjects

18 27 to 60-year-old male and female patients with rheumatoid arthritis, defined according to the American Rheumatism Association's criteria [Ropes, 1958, Slesberg 1964], Felty's syndrome, psoriatic arthritis, systemic lupus erythematosus, Sharp's syndrome and Soderb's disease were studied (table I).

The diagnosis of the rheumatic diseases was based on the history, clinical signs and symptoms, and on the following laboratory findings: ESR, C-reactive protein test, mucoproteins, RA test, Waaler-Rose reaction, LE cells, complement, DNA antibodies, antinuclear factor anti-DNA antibodies, serum protein electrophoresis, IgA, IgM, IgG determination.

All patients studied were afebrile.

16 healthy male and female controls were also studied after careful screening of their clinical conditions. Informed consent was obtained from all patients and controls.

#### Etiocholanolone Test

The method of Vogel *et al.* [1967] was used with slight modification [Cambiaghi and Paina,

Medicine, Fujita-Gakuen University Hospital, and Dr Noriyoshi Hara and Dr Tetsuma Igarashi of the Department of Pediatrics, Yokohama Kairyu Hospital, who made it possible for us to obtain blood of patients with hereditary methemoglobinemia. We also express our thanks to Dr Matsukawa for his advice. This work was partially supported by grants from the Ministry of Education, Science and Culture of Japan and the Intractable Diseases Division, Public Health Bureau, Ministry of Health and Welfare, Japan

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Table II. Changes in bone marrow reserve in 16 healthy controls and in 18 rheumatic patients (10 of them with rheumatoid arthritis)

	Baseline counts of granulocytes/ $\mu$ l	Granulocytes/ $\mu$ l after 15 h	$\Delta$ G
<i>Healthy controls</i>			
1	5,100	9,100	4,000
2	5,600	10,100	4,500
3	4,900	7,900	3,000
4	5,200	8,200	3,000
5	5,600	10,100	4,500
6	5,500	8,900	3,400
7	5,300	8,900	3,600
8	5,000	7,900	2,900
9	5,100	9,100	4,000
10	5,200	8,200	3,000
11	5,900	9,900	4,000
12	4,900	7,900	3,000
13	5,300	9,700	4,200
14	5,200	8,200	3,000
15	5,000	8,600	3,600
16	5,600	10,100	4,500
Mean	5,286		3,631
SD	$\pm 306$		$\pm 617$
CV	5.80		16.99
<i>Rheumatic patients</i>			
1 Rheumatoid arthritis	11,000	13,700	2,700
2 Rheumatoid arthritis	6,200	11,600	5,400
3 Rheumatoid arthritis	6,200	11,600	5,400
4 Rheumatoid arthritis	3,400	9,200	5,800
5 Rheumatoid arthritis	4,400	5,200	800
6 Rheumatoid arthritis	4,100	5,800	1,700
7 Rheumatoid arthritis	8,800	10,000	1,200
8 Rheumatoid arthritis	4,600	5,900	1,300
9 Rheumatoid arthritis	5,200	9,000	3,800
10 Rheumatoid arthritis	3,900	5,000	1,100
11 Felty's syndrome	3,000	4,600	1,600
12 Felty's syndrome	3,400	4,900	1,500
13 lupus erythematosus	4,200	6,000	1,800
14 lupus erythematosus	3,900	5,200	1,300
15 lupus erythematosus	3,600	5,400	1,800
16 Sharp's syndrome	3,800	7,200	3,400
17 Sudeck's disease	3,400	11,200	7,800
18 psoriatic arthritis	8,800	12,000	3,200

Table I. Clinical data in rheumatic patients. Patient characteristics

No.	Initials	Sex	Age	Diagnosis	Spleen enlargement
1	G.C.	f	52	rheumatoid arthritis	±
2	M.N.	f	47	rheumatoid arthritis	-
3	C.M.	f	51	rheumatoid arthritis	-
4	F.T.	f	60	rheumatoid arthritis	-
5	Z.T.	f	33	rheumatoid arthritis	+
6	R.E.	f	50	rheumatoid arthritis	+
7	P.R.	f	27	rheumatoid arthritis	+
8	L.G.	f	29	rheumatoid arthritis	±
9	P.L.	m.	40	rheumatoid arthritis	-
10	T.F.	m.	55	rheumatoid arthritis	±
11	G.F.	f	27	Felty's syndrome	++
12	F.C.	f	57	Felty's syndrome	++
13	C.M.	f	28	lupus erythematosus	+
14	M.G.	f	27	lupus erythematosus	+
15	C.L.	f	33	lupus erythematosus	+
16	A.G.	f	60	Sharp's syndrome	-
17	R.M.	m.	37	Soderck's disease	-
18	P.R.	f	27	psoriatic arthritis	-

- = No spleen enlargement    + = 10-mm enlargement    ++ = 20-mm enlargement.

1978, 1979] Exactly at midnight, each individual received an intramuscular injection of 0.1 mg/kg of a 1% solution of etiocholanolone in propylene glycol. At 3 p.m. on the previous day and 15 h after the injection, peripheral granulocyte were counted in duplicate in a Bürker chamber and differentials were determined by counting at least 200 cells in smears stained according to May Grünwald-Giemsa.

The difference ( $\Delta G$ ) between the baseline count and the granulocyte count obtained 15 h after the etiocholanolone injection was calculated. In 1 case (No 18) the test was done five times, i.e. before, during (two tests at 1-month intervals) and after treatment with penicillamine at a dose of 600 mg/day for 3 months.

## Results

The results are summarized in table II which shows the individual data, the mean

with standard deviations (SD) and the coefficient of variation (CV) of the baseline granulocyte counts and  $\Delta G$  in healthy controls and patients with rheumatic diseases.

In healthy controls, the mean baseline granulocyte count was  $5,286 \pm 306$  and the CV was 5.80. In rheumatic patients, the mean baseline granulocyte count was  $5,105 \pm 2,263$  and the CV was 44.32. These baseline counts do not differ significantly ( $t = 0.31$  p n.s.). In healthy controls, the mean value of  $\Delta G$  was  $3,631 \pm 617$  and the CV was 16.99. In rheumatic patients, the mean value of  $\Delta G$  was  $2,644 \pm 1,941$  and the CV was 73.43. These mean values do not differ significantly ( $t = 1.94$  p n.s.).

However if only the subjects with rheumatoid arthritis (cases 1-10) are consid

Table II. Changes in bone marrow reserve in 18 healthy controls and in 18 rheumatic patients (10 of them with rheumatoid arthritis)

	Baseline counts of granulocytes $\mu$ l	Granulocytes $\mu$ l after 15 h	$\Delta$ C
<i>Healthy controls</i>			
1	5 100	9 100	4,000
	5,600	10,100	4,500
3	4,900	7,900	3,000
4	5,200	8,200	3,000
5	5,600	10 100	4,500
6	5,500	8,900	3 400
7	5 900	8,900	3 400
8	5,000	7,900	2,900
9	5 100	9 100	4,000
10	5,200	8,200	3,000
11	5 900	9,900	4,000
12	4 900	7 900	3,000
13	5,500	9 700	4,200
14	5,200	8,200	3,000
15	5,000	8,600	3,600
16	5,600	10,100	4,500
Mean	5,284		3,631
SD	$\pm$ 306		$\pm$ 417
CV	5.80		16.99
<i>Rheumatic patients</i>			
1 Rheumatoid arthritis	11,000	13,700	2,700
2 Rheumatoid arthritis	6,200	11,500	5 400
3 Rheumatoid arthritis	6,200	11,600	5,400
4 Rheumatoid arthritis	3 400	9,200	5,800
5 Rheumatoid arthritis	4,400	5,200	800
6 Rheumatoid arthritis	4,100	5,800	1 700
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8 Rheumatoid arthritis	4,600	5,900	1,300
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17 Sudeck's disease	8,800	12,000	3,200
18 psoriatic arthritis			

Table II (continued)

	Baseline counts of granulocytes/ $\mu$ l	Granulocytes/ $\mu$ l after 15 h	$\Delta G$
Mean of rheumatic patients	5,105		2,644
SD	$\pm 2,263$		$\pm 1,941$
CV	44.32		73.43
Mean of rheumatoid arthritis patients	5 780		2,520
SD	$\pm 2,412$		$\pm 1,851$
CV	41.73		73.47

Table III. Bone marrow reserve in the patient with psoriatic arthritis, examined before, during and after treatment with penicillamine

	Baseline counts of granulocytes/ $\mu$ l	Granulocytes/ $\mu$ l after 15 h	$\Delta G$
Before the treatment	8,800	12,000	3,200
During the treatment	5,000	6,600	1,600
During the treatment	4,600	5 800	1,200
During the treatment	4,500	5 700	1,200
After the treatment	2,800	3 700	900

ered, it is observed that, while there was no statistically significant difference in the baseline granulocyte counts in healthy controls (mean  $5,286 \pm 306$  with a CV of 5.80) and rheumatoid arthritis patients (mean  $5,780 \pm 2,412$ , with a CV of 41.73  $t = 0.817$  *p* n.s.) the mean value of  $\Delta G$  in these patients ( $2,520 \pm 1,851$  with a CV of 73.47) is significantly reduced as compared to the mean  $\Delta G$  observed in healthy controls ( $3,631 \pm 617$  with a CV of 16.99  $t = 2.23$   $p < 0.05$ ).

In the last patient (case 18) who had psoriatic arthritis, the bone marrow was examined five times, i.e. before, during (once a month during 3 months) and after treatment with penicillamine (table III). At first the bone marrow reserve was normal, but

there was a progressive reduction in the bone marrow reserve in the subsequent tests, while the peripheral granulocyte count remained normal.

### Discussion

The etiocholanolone test showed that the rheumatoid arthritis patients had a reduced bone marrow granulocyte reserve, which could be attributed to various factors.

First of all it is frequently found that such patients have previously taken drugs with potential marrow toxicity [Pisciotto *et al.*, 1958; Corcos *et al.* 1964; Huguley 1964; Balne and Huskisson, 1972; Satta 1973].

Our results disagree with Hall *et al* [1973] who failed to find a difference between the marrow reserve of patients with rheumatoid arthritis and controls. An explanation is that while our rheumatoid arthritis patients were on therapy those of the series of Hall *et al* probably were not.

In addition, these patients may have hypersplenism the increased sequestration or waste of granulocytes by the spleen produces a compensative hyperplasia of these cells by the bone marrow [Falk and Sacchetti 1967] the bone marrow reserve may be sufficient to maintain a normal number of circulating granulocytes, but not to maintain a normal bone marrow reserve. Finally the possibility that circulating anti-granulocyte autoantibodies exist cannot be excluded from an explanation of the reduction of the bone marrow reserve in rheumatoid arthritis patients [Barnes *et al* 1971].

As regards the other cases with Felty's syndrome and systemic lupus erythematosus, our data, even though limited indicate a tendency to a reduction of the bone marrow reserve also in these cases it could be attributed to splenomegaly with consequent hypersplenism and/or to the presence of circulating autoantibodies [Hume *et al* 1964].

The bone marrow reserve was normal in the patient with Sharp's syndrome (case 16) and in the patient with Sudeck's disease (case 17), but more cases are needed to confirm these results.

In the last patient, who had a psoriatic arthritis, a progressive reduction in the bone marrow reserve after penicillamine treatment was observed, this confirms that the drug has a toxic effect on the bone marrow [Corcos *et al* 1964 Balne and Huskisson, 1972].

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	Baseline counts of granulocytes/ $\mu$ l	Granulocytes/ $\mu$ l after 15 h	$\Delta G$
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During the treatment	5,000	6,600	1,600
During the treatment	4,600	5,800	1,200
During the treatment	4,500	5,700	1,200
After the treatment	2,800	3,700	900

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## Chronic Lymphoid Leukemia

Clinical Observations about Organomegaly

21

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**Key Words.** CLL. Adenosplenomegaly. Leucocytes in CLL.

**Abstract.** 223 cases with chronic lymphoid leukemia (CLL) were subjected to a prospective study on the presence of adeno- and splenomegaly at diagnosis and their subsequent variation. Subjects with no initial organ involvement were usually female (76%) and of old age (mean 69.2 years). Adenopathy or combined lymph node and spleen enlargement were inversely proportional to the mean age at diagnosis. Subsequent organ enlargement was noted in 11.7% of patients with no initial organomegaly, splenomegaly in 15% of patients with adenopathy only and adenopathy in 15% of patients with splenomegaly only. Except in the few cases with leukopenia, particularly high leukocyte levels were noted in patients with splenomegaly (with or without adenopathy). Anemia at diagnosis was not related to the degree of organ enlargement. It is suggested that qualitative and also quantitative differences in organomegaly in CLL merit further study to establish their underlying mechanisms. CLL must be seen as something more complex than the simple mechanical expression of progressive lymphocyte accumulation.

Recent cytological research has led to the differentiation of individual forms of chronic lymphoid leukemia (CLL): hairy cell [18], T lymphocyte [15] and prolymphocytic leukemia [4, 11].

A clinical distinction into 'pure nodular' forms, with no adenopathy nor splenomegaly, and 'pure splenomegalic' forms, with splenomegaly only, had already been established and its significance investigated

by careful assessment of the relevance of adenopathy and spleen enlargement in CLL [1, 13, 14, 20].

Our observations on CLL have reinforced our opinion that clinical differences in these organomegalies are related to biologically important events. We also feel that the clinical approach to CLL should appreciate and quantitatively evaluate the extent of organ enlargement.

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## Chronic Lymphoid Leukemia

### Clinical Observations about Its Natural Progression

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Ospedale Maggiore di S. Giovanni Battista della Città di Torino, Turin

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Our observations on CLL have reinforced our opinion that clinical differences in these organomegalies are related to biologically important events. We also feel that the clinical approach to CLL should appreciate and quantitatively evaluate the extent of organ enlargement.

## Material and Methods

Diagnosis of CLL was based on persistent blood and bone marrow lymphocytosis not otherwise explicable. Lymphocytosis is not necessarily absolute, as in the case of leukopenic CLL [13]. Patients with hairy cell leukemia or suspected malignant lymphoma were excluded.

Patients were initially studied for phytohemagglutinin (PHA) response (1970). Later spontaneous rosette formation with sheep RBC (E-rosettes, 1972) and detection of surface membrane immunoglobulin receptors (Smig, 1973) were used.

Our series consists of 223 patients observed from diagnosis in our hospital up to December 31, 1978. Organomegaly was the subject of particular attention, and was studied prospectively. Details of organ enlargement were entered on a printed body diagram to provide comparable, objective data. Clinical observations were always made by members of the same staff.

Organ enlargement was assessed empirically from + to +++++. Both the number of sites and node volumes were considered in adenopathy. For splenomegaly we discarded linear evaluation in centimeters, distorted by vertical or horizontal growth, since we feel that volume is more important in clinical appraisal. The spleen size was graded as follows: + = constantly palpable; ++ = occupying almost all the left hypochondrium to the transverse umbilical line (TUL); +++ = extending beyond TUL to midline; ++++ = extending beyond midline, with its lower pole in the pelvis. Liver volume was left out of account, because it may be altered by hepato-

pathies or cardiac failure, specially in aged patients.

Patients in whom lymph node and spleen enlargement was of the same degree were defined as "balanced"; the term "unbalanced" was used for patients in whom lymph node enlargement exceeded splenomegaly or vice versa.

Intermittent chlorambucil (2.5-15 mg/day) was given where necessary and cyclophosphamide (50-150 mg/day) in patients with chlorambucil intolerance, resistance and/or thrombocytopenia. Radiotherapy was rarely employed, mainly for splenomegaly.

## Results

223 patients have been observed, with 71 deaths recorded. The number includes 121 (54%) males and 102 (46%) females. Mean age at presentation was 64.4 years (63.3 years for males, 65.8 years for females). Median survival was 76 months (64 months for males, 81 months for females). Superficial lymph node and spleen involvement at the time of diagnosis of all the patients is shown in table I.

### Organomegaly and Sex

A male predominance was noted in nearly all types of organ enlargement; the most notable exception is the group of patients

Table I. Adeno- and splenomegaly at diagnosis (223 cases)

	Cases		Males		Females	
	n	%	n	%	n	%
No adenosplenomegaly	42	19	11	24	31	76
Adenomegaly only	49	22	29	59	20	41
Splenomegaly only	33	15	24	73	9	27
Adeno- and splenomegaly (balanced)	55	25	31	56	4	44
Adeno- and splenomegaly (unbalanced)	44	19	26	59	18	41

who did not exhibit detectable enlargement with 24 males and 4 females. This female predominance has been observed by *Cocur et al* [1] French workers cited by them.

### Organomegaly and Age

*Cocur et al* [7] observed that adenomegaly was more common in older subjects. In our group of 23 patients the mean age was 69 years in the 4 patients with neither adenopathy nor splenomegaly.

We examined the mean age at presentation within groups with different organ involvement, supposing that those with minor organ enlargement would be the youngest if delay in diagnosis was of major importance. On the contrary table II shows that age and size of nodes are inversely related in cases of simple adenopathy and in combined adenopathy and splenomegaly. This is not so when only splenomegaly is present: spleen size rated + at 64.3, ++ at 63.4, +++ at 65.1, ++++ at 71.5 years of mean age respectively.

### Changes in Organ Enlargement and Spleen Enlargement

Changes in organ enlargement during the course of the disease were followed in 86 patients observed for more than 24 months since diagnosis. The most prominent organomegaly noted during the first 3 months since diagnosis was used as start pattern; their variations (appearance or increase of adenomegaly and or splenomegaly) are summarized in table III. The data from table III suggest that the pattern of organomegaly alters little during the course of CLL. If not

Table II. Comparison of age and organomegaly at diagnosis: some patients were gathered in group V as they were considered of equal clinical significance

Group	Grading	Number of cases	Age mean value
I No adenomegaly	-	42	69.2
II Adenomegaly	+	79	66.7
III Adenomegaly	+	9	59.6
IV Adenomegaly	++++	11	58.3
V	Adenomegaly	29	65.8
	Splenomegaly	8	
	Adeno-splenomegaly	30	
VI Adeno-splenomegaly	+	30	63.4
VII Adeno-splenomegaly	++	21	61.0
VIII Adeno-splenomegaly	+++	4	59.0

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Table IV. Cytotoxic therapy in re

## Groups and grading

No adenomegaly				
Adenomegaly				
+		4		
+				
++				1
+++		1		
++++				75
Splenomegaly				
+	16	16	10	18
++		50	25	5
+++	1	50	26	12
++++			25	75
Adeno-splenomegaly				
+	18	47	17	18
++		30	40	40
+++		14	43	43

*CLL with Leukopenia*

A WBC count of  $<4.0 \times 10^9/l$  was noted in 4 patients (all males) with + + + + + splenomegaly. They were nevertheless, classed as CLL on the basis of frank and persistent lymphocyte predominance in blood normal to hypercellular bone marrow with 52-70% lymphocytes (except in the 2 splenectomized subjects) 1-20% rosettes.

Two splenectomized patients, with no evidence of hairy cell leukemia, had WBC counts of  $1.8-2.5 \times 10^9/l$  and  $1.6-4.0 \times 10^9/l$ , respectively. Their bone marrow was normocellular (lymphocytes 20%) and hypercellular (lymphocytes 89%). E rosettes represented 6 and 1%. The spleens were removed after 1 month and 13 months, respectively and weighed 3 000 and 1,600 g. In each case, there was a histo-

logical finding of diffuse lymphocyte infiltration of the spleen and the portobiliary spaces.

In the first case, the WBC count rose to  $34.0 \times 10^9/l$ , with neutrophils (N) 10%, rosettes 13%, 22 months after surgery. The marrow was hypercellular with lymphocytes (L) 53%. No adenopathy appeared in a period of 59 months after splenectomy.

In the second case, the leukocyte count rose to  $150.0 \times 10^9/l$ , with N 1%, E rosettes 6%, SmIgM 90% and SmIgG 13%, 7 months after surgery. The marrow was hypercellular with L 93%. No adenopathy appeared in the 14 months prior to death.

*Organomegaly and Leukocyte Count*

Hansen [13] found that the highest WBC counts were usually associated with spleno-



Table III Variation of adeno- and/or splenomegaly in 86 cases observed for more than 24 months (see text)

Grading	Number of patients	Number of cases and grading of						Mean value of observation, months
		appearance of			increase of			
		adeno- megaly	spleno- megaly	%	adeno- megaly	spleno- megaly	%	
No megaly	17	1 + and 1 + and	+ ++	12	-	-	-	41
Adenomegaly only	27	-	4 +	15	5	-	18	47
Splenomegaly only	20	3 +	-	15	-	1 +++	5	38
Unbalanced megaly								
Adenomegaly predominant	12	-	-		1 +++ and 2 +++	+++ 3 ++	33	47
Splenomegaly predominant	10	-	-		1 ++ 1 +++	-	20	48

present at diagnosis, their subsequent appearance was noted in only 11.7% of cases. This tendency to maintain the status quo is also evident from the data of *Coeur et al* [7 subsequent adenopathy in 3/17 cases] *Rai et al* [16.3 subsequent adenopathies and 7 splenomegalies in 22 cases] and *Binet et al* [1 subsequent organomegaly in 8/48 cases]. An explanation has still to be found for the observation by lymphangiography of retroperitoneal node enlargement in patients with no superficial adenopathy [1 personal data].

Spleen enlargement appeared in only 14.8% of subjects with adenopathy and adenopathy in 15% of those with splenomegaly. In cases of "unbalanced" enlargement increases in size of the less involved organ were noted in 33% of cases for splenomegaly and 20% for adenopathy. *Binet et al* [1] also noted subsequent splenomegaly in only 4/33 subjects with adenopathy (= 11.1%) and subsequent adenopathy in only 1/8 with splenomegaly (= 12.4%).

Two points would thus seem to emerge with regard to changes in organomegaly after diagnosis: (1) the initial picture is not likely to change: patients with adenopathy alone rarely develop splenomegaly and vice versa; (2) patients without initial organomegaly rarely develop subsequent organ enlargement.

#### Organomegaly and Treatment

We separated untreated cases from those subjected to very light treatment (short courses, 1 year or more apart), medium treatment (one or more well-spaced courses per year) and heavy more or less continuous treatment. The details are shown in table IV.

Most patients without detectable or with very mild adeno- and/or splenomegaly had no medium or heavy treatment. This suggests that cytostatic treatment plays a minor role in bringing about changes in organ enlargement. *Binet et al* [1] found that cytostatic treatment was not needed in 44/124 cases (= 35.4%).

Table IV Cytostatic therapy

Groups and grading

No adeno-splenomegaly

Adeno-splenomegaly

++  
+++  
++++

Splenomegaly

++  
+++  
++++

Adeno-splenomegaly

++  
+++

C			
1 + m - r			
1 m			
5		4	
	45		8
	19	1	18
	1		55
		5	75
36	36	10	18
	30	25	25
1	30	26	12
		25	75
18	47	17	18
	20	40	40
	14	43	41

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logical finding of diffuse lymphocyte infiltration of the spleen and the portobiliary spaces.

In the first case the WBC count rose to  $34.0 \times 10^9/l$ , with neutrophil (N) 10%, E rosettes 13%, 2 months after surgery. The marrow was hypercellular with lymphocytes (L) 53%. No adenopathy appeared in a period of 59 months after splenectomy.

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### Organomegaly and Leukocyte Count

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Table V Mean values WBC count at diagnosis with respect to organomegaly 4 leukopenic cases are excluded

Groups and grading	Number of cases	WBC mean values ( $\times 10^9/l$ )
I No adeno-splenomegaly	42	26.70
II { Adenomegaly + Splenomegaly + Adeno-splenomegaly + }	67	34.25
III Adenomegaly ++/+++/+++++	20	32.75
IV Splenomegaly ++/+++/+++++	21	108.00
V Adeno-splenomegaly ++/+++	25	175.90
VI Adenomegaly predominant	23	54.04
VII Splenomegaly predominant	21	83.95

megaly Subsequently it was observed that prolymphocytic CLL, which is usually accompanied by massive spleen enlargement, presents with high WBC [4 5 11] Table V compares organomegaly and WBC values at presentation in 219 cases, the 4 leukopenic patients being excluded

Two groups of mean WBC are apparent one with a maximum of  $54.0 \times 10^9/l$  the other with means of  $83.9$  to  $175.9 \times 10^9/l$  The highest mean value of WBC was noted in patients with marked adenopathy and splenomegaly (group V) followed by group IV with massive spleen enlargement only and group VII with predominant splenomegaly and modest adenopathy

Student's *t* test showed a highly significant mean difference ( $p < 0.01$ ) between groups I and IV I and V III and IV III and V II and IV II and V The difference between groups III and VII was significant at  $p < 0.05$

CLL with splenomegaly may be frequently associated with a higher WBC count in contrast to patients with simple adenopathy Splenomegalic dominance is associated with higher WBC values even in the cases of

unbalanced enlargement. It thus appears that enhanced accumulation of lymphocytes in blood is brought about by marked enlargement of the spleen.

#### *Organomegaly and Anemia*

Anemia at the time of diagnosis is generally equated with poor prognosis [6 13 14 20] as in our patients (median survival 25 months when RBC less than  $3 \times 10^{12}/l$ , 81 months if higher) Rai *et al.* [16] agree with regard to prognosis, but view anemia (and thrombocytopenia) as the advanced stages (III and IV) of a progressive postorganomegalic accumulation of lymphocytes. If this were true, anemia at the time of diagnosis should be more frequent in patients with most pronounced organomegaly In our series this, however was not the case In fact, the proportion of patients with anemia ( $RBC < 3.0 \times 10^{12}/l$ ) reached 11% when adenosplenomegaly was absent or moderate (+/++) and not far more (16%) when organomegaly was more important (+++/++++)

It can be assumed therefore that anemia does not necessarily follow node or

spleen enlargement, and is already a poor prognostic sign when present at the time of diagnosis. Subsequent anemia may have a different pathogenesis, e.g. progressive marrow hypocellularity caused by wasting cytostatic treatment, infections, etc.

### *Differential Leukocyte Count*

Lymphocytosis usually persists in CLL even after normalization of the WBC count as a result of treatment. This phenomenon has not received the attention which we feel it deserves.

9 (41%) of our patients never regained a normal WBC level (intensive therapy not required or impossible). Of the 132 remaining patients, 7 (5%) displayed a neutrophil percentage around 50%, while in 11 cases (85%) lymphocyte prevalence persisted despite the normal WBC count, even when smudge cells were excluded. Of the 13 patients (10%) with a neutrophil percentage persistently higher than 50%, 1 had Richter's syndrome, 4 carcinoma and 2 a progressive IgM increase. We therefore consider that a normal differential count in CLL in remission must be regarded with suspicion.

The persistence of a lymphocyte prevalence is particularly striking when CLL is compared with macroglobulinemia. In the majority of cases of this latter disease treatment with chlorambucil provokes a rapid and profound lymphocytopenia with minor suppression of myelopoiesis.

### *Discussion*

Our personal observations confirm results of previous studies [1-16] suggesting that CLL does not often change its clinical

pattern. This constance cannot be ascribed to treatment, and it was noted also in the cases with minor organomegaly in which intensive treatment was not required. In our opinion this observation suggests that CLL,

in many cases, has not necessarily a progressive course. Where adenopathy only or adenopathy and splenomegaly were present, at diagnosis was relatively lower in patients with most pronounced organ enlargement (table II). This could mean that CLL presenting with considerable organ enlargement is not the outcome of an insidious progression but that the disease presents with different features.

There are four points in favour of this view: (1) CLL with simple adenopathy or splenomegaly at diagnosis has rarely (15%) changed; (2) in CLL with 'unbalanced' spleen and lymph nodes enlargement the unbalancement does not change; (3) our two splenectomized subjects displayed a marked increase in blood and marrow lymphocytes, but no adenopathy; (4) anemia at presentation is not necessarily more frequent in patients with a greater degree of organomegaly.

These findings are in conflict with the view that CLL is progressively expensive, e.g. onset without enlargement, followed by adenopathy and then splenomegaly ending with anemia and thrombocytopenia, as the expression of progressive lymphocyte accumulation [16].

CLL can be divided clinically into 'benign' and 'malignant' forms [2]. Benign forms, with or without minor organomegaly are rarely progressive. The persistence (clearly not due to therapy) of single or predominant enlargement of lymph nodes or spleen suggests that some different biological mechanisms can be involved.

Different behaviour of leukocyte number noted by Hansen [13] and by us in our splenomegalic patients support this view. Already Di Guglielmo [8] suggested that splenomegalic forms are not true CLL, but histioleukemia. The recognition of hairy cell leukemia has itself led to a breakdown in the conventional definition of CLL.

Differences in B lymphocyte maturity in CLL [3-17] can modulate the virtual inability of B lymphocytes to leave the blood stream [9] and the lymphocyte membrane as the passage through the post-capillary venules [10-19] or the expression of surface Ig [12] are concerned. The relationship between different biological markers of lymphocytes and the different patterns of organomegaly are worthy of further investigation.

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## Transient Aplastic Crisis in Hereditary Spherocytosis

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**Key Words.** Aplastic crisis Hereditary spherocytosis Infection Phagocytosis

**Abstract.** In this study the case of a child affected with hereditary spherocytosis, who presented with a presumably viral infection induced transient aplastic crisis, is described. The bone marrow aspirate showed phagocytosis of erythrocytes and nucleated cells by reticular cells. The possible pathogenetic role of bone marrow phagocytosis in aplastic crises in patients with haemolytic anaemias is discussed

### Introduction

It is well known that, in patients with chronic haemolytic anaemias, there may occur intercurrent episodes of medullary aplasia of brief duration and spontaneous resolution (1, 2, 4-8, 11-13). The aetiology of such crises is not yet clear. They are frequently connected with infective episodes, particularly of viral origin.

Here we describe the case of a child affected with hereditary spherocytosis, who manifested a transient aplastic crisis with numerous examples of phagocytosis in the bone marrow.

### Case Report

We present a 7-year-old girl, with negative family history who has, in the past, had some epi-

sodes of pallor with mild jaundice. In another hospital, she had recently had 9 teeth extracted under general anaesthesia with halothane. At this time, clinical examination showed scleral subicterus, together with mild hepatomegaly and spleen palpable 2 cm below the costal margin (Hb, 10.8 g/dl; platelets,  $250 \times 10^9/l$ ; WBC  $8.4 \times 10^9/l$ ; neutrophils, 41%; reticulocyte count, 11.5%; total serum bilirubin, 2.6 mg%; direct, 0.2 mg%; Coombs' direct test, negative). 1 week after surgery an infective episode occurred, probably viral in origin, with intermittent pyrexia (39-40°C) for 3 days. Chest X-ray, urine and blood culture were all negative. At this time the blood picture altered considerably: Hb 6.6 g/dl; reticulocytes, 2.3%; bilirubinemia, 1.1 mg%. For this reason the child was transferred to the Pediatric Clinic, Milan University. She presented with mild jaundice and hepatosplenomegaly. The investigations clearly showed a medullary insufficiency (Hb, 7.1 g/dl; Ht, 18.2%; RBC,  $2.170 \times 10^{12}/l$ ; MCV 82  $f_m^3$ ; Reticulocyte count, 1.2%; WBC,  $1.4 \times 10^9/l$ ; neutrophils, 16%; platelets,  $97 \times 10^9/l$ ; total serum bilirubin, 1.04 mg%, di-

rect, 0.2 mg% 1. A more complete h study resulted in diagnosis of hereditary spherocytosis: spherocytes were demonstrated on blood smear; the erythrocytes showed osmotic resistance (haemolysis: 0.55% NaCl and 94.8% in 0.30% NaCl caused autohaemolysis (30.3% in haemolysis, 19.9% in presence of glucose, presence of ATP). Hb electrophoresis: logical Hb: Hb A<sub>2</sub> 9%, G6PD: Ph<sub>n</sub> normal.



Fig. 1 A Phagocytosis of three red cells by reticular cell. B Phagocytosis of one red cell and some nuclear material. C Phagocytosis of nuclear material and one unsegmented nucleus of granulocyte. D Giant proerythroblast.



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rect, 0.2 mg<sup>g</sup>). A more complete haematological study resulted in a diagnosis of hereditary spherocytosis: spherocytes were demonstrated in the peripheral blood smear; the erythrocytes showed reduced osmotic resistance (haemolysis of 25.4% in 0.55% NaCl and 94.8% in 0.30% NaCl) and increased autohaemolysis (30.3% in absence of substrates, 19.9% in presence of glucose, 14.7% in presence of ATP); Hb 11 g/dl, reticulocytes, non-pathological; Hb Hb A<sub>2</sub> 9%, G6PD PK, normal blood

Direct and indirect Coombs test negative. The marrow aspirate showed diminished cell count, with striking hypoplasia in the erythroblastic series, particularly the more mature cells; myeloid:erythroid ratio: 5:1; scanty megakaryocytes; large cells with loose chromatin and 1-2 nucleoli (giant proerythroblasts); numerous reticular cells with cytoplasmic inclusions comprising whole red cells, their fragments, granulocytes and other nucleated cells (fig 1). All the investigations aimed



Fig. 1 A Phagocytosis of three red cells by a reticular cell. B Phagocytosis of one red cell and some nuclear material. C Phagocytosis of nuclear material and one unsegmented nucleus of granulocyte. D Giant proerythroblast.

at establishing the aetiology of the intercurrent infective illness proved negative: urine, blood and stool culture, Widal test, Wright test, tubercollin test, antistreptolysin O titre, *Toxoplasma* antibodies and virological studies (monotest cytomegalovirus, coxsackievirus and rubella antibodies). 3 blood transfusions proved necessary. The temperature returned to normal after 1 day in hospital. In the subsequent 4-5 days the haematological situation spontaneously improved, with increase of reticulocytes, platelets and granulocytes. Hb remained stable at about 10 g/dl. Another marrow aspirate, performed 6 days after the first one, was normocellular with normal progression of erythroid, myeloid and megakaryocytic series with no evidence of phagocytosis. 11 months later the child had a splenectomy and she is, at the present time in optimal general and haematological conditions.

## Discussion

In 1948 *Ovren* [11] first described a picture of transient aplastic crises in patients with hereditary spherocytosis. In these cases, and in several subsequent reports in various haemolytic anaemias [14-8 12] the haematological picture is characterized by anaemia, reticulocytopenia, reduction of bilirubinaemia, frequent granulocytopenia and lowered platelet count. These crises arise for the most part following an episode of viral infection and spontaneously resolve in 7-10 days.

The pathogenesis of this condition is not yet clear. The infective agent could produce a cytotoxic damage in the bone marrow either directly or by an immunological mechanism. An infection-induced folate deficiency has to be considered even if we did not check the serum folate levels in our patient this hypothesis may be excluded owing to the absence of the typical bone marrow picture (presence of megaloblastic cells) (4)

The possible aetopathogenetic role of halothane cannot be excluded, but it seems quite unlikely as there are no reports of halothane-induced bone marrow damage while the correlation with infectious disease is well known.

The bone marrow is characterized by erythroid hypoplasia, the presence of giant proerythroblasts (35-40  $\mu$ m) and, at times, by diminution or altered maturity in the cells of the myeloid series and of the megakaryocytes.

*Sansone* [12] in 1955 described, in a case of spherocytosis with aplastic crisis, aspects of medullary erythrophagocytosis apart from the monocytic series. No other author to our knowledge has since reported a similar medullary phenomenon. Our case described here not only confirms the presence of phagocytosis of red cells, but also demonstrates phagocytosis of granulocytes and other nucleated elements. This appearance was no longer present in the examination carried out after only 5 days.

Erythrophagocytosis in the bone marrow is a physiological mechanism in the breakdown of the red cells, which is not seen in the usual marrow aspirate but is demonstrated only at biopsy with special staining techniques [9 10] or by electron microscopy. The discovery of pictures of phagocytosis in materials obtained by simple aspiration can therefore, be considered the evidence of a much more intensive activity in the bone marrow.

Erythrophagocytosis has been observed in routine peripheral smears of patients with haemolytic anaemias (especially the autoimmune ones) leukaemias, Hodgkin's disease and lymphosarcomas and in the bone marrow aspirates of patients with autoimmune anaemias (2, 13) or transient histiocytosis

associated with military tuberculosis or viral infection (3).

Studies on erythrophagocytosis *in vitro* performed incubating vital phagocytes with chemically injured or antibody-coated red cells showed that the antibody-coated ones adhere more easily to the phagocytes and are more rapidly ingested (2).

It is not possible, on the basis of our experience, to state precisely whether the phagocytosis present in the bone marrow at the time of the aplastic crisis is a coincidence or whether it is directly responsible for the medullary damage, even if we can reasonably presume an enhancing of this process owing to the virus-induced immunological alterations.

We believe, however that this communication may serve to promote a study in depth (not only aspirates, but bone marrow biopsy immunological picture, number of the CFU-E, erythropoietin levels) of cases of aplastic crisis during the course of haemolytic anaemias. Only thus will it be possible to clarify the various pathogenetic aspects of a phenomenon of which there is much to be learned.

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at establishing the aetiology of the intercurrent infective illness proved negative; urine, blood and stool culture, Widal test, Wright test, tuberculin test, antistreptolysin O titre, *Toxoplasma* antibodies and virological studies (monotest cytomegalovirus, coxsackievirus and rubella antibodies). 3 blood transfusions proved necessary. The temperature returned to normal after 1 day in hospital. In the subsequent 4-5 days the haematological situation spontaneously improved, with increase of reticulocytes, platelets and granulocytes. Hb remained stable at about 10 g/dl. Another marrow aspirate, performed 6 days after the first one, was normocellular with normal progression of erythroid, myeloid and megakaryocytic series with no evidence of phagocytosis. 11 months later the child had a splenectomy and she is, at the present time in optimal general and haematological conditions.

## Discussion

In 1948 *Owren* [11] first described a picture of transient aplastic crises in patients with hereditary spherocytosis. In these cases, and in several subsequent reports in various haemolytic anaemias [14-8, 12] the haematological picture is characterized by anaemia, reticulocytopenia, reduction of bilirubinaemia, frequent granulocytopenia and lowered platelet count. These crises arise for the most part following an episode of viral infection and spontaneously resolve in 7-10 days.

The pathogenesis of this condition is not yet clear. The infective agent could produce a cytolytic damage in the bone marrow either directly or by an immunological mechanism. An infection induced folate deficiency has to be considered even if we did not check the serum folate levels in our patient, this hypothesis may be excluded owing to the absence of the typical bone marrow picture (presence of megaloblastic cells) (4).

The possible aetiopathogenetic role of halothane cannot be excluded, but it seems quite unlikely as there are no reports of halothane induced bone marrow damage, while the correlation with infectious disease is well known.

The bone marrow is characterized by erythroid blastopenia, the presence of giant proerythroblasts (35-40  $\mu$ m) and, at times, by diminution or altered maturity in the cells of the myeloid series and of the megakaryocytes.

*Sansone* [12] in 1955 described, in a case of spherocytosis with aplastic crisis, aspects of medullary erythrophagocytosis apart from the monocytic series. No other author to our knowledge has since reported a similar medullary phenomenon. Our case described here not only confirms the presence of phagocytosis of red cells, but also demonstrates phagocytosis of granulocytes and other nucleated elements. This appearance was no longer present in the examination carried out after only 6 days.

Erythrophagocytosis in the bone marrow is a physiological mechanism in the breakdown of the red cells, which is not seen in the usual marrow aspirate but is demonstrated only at biopsy with special staining techniques [9, 10] or by electron microscopy. The discovery of pictures of phagocytosis in materials obtained by simple aspiration can therefore be considered the evidence of a much more intensive activity in the bone marrow.

Erythrophagocytosis has been observed in routine peripheral smears of patients with haemolytic anaemias (especially the autoimmune ones), leukaemias, Hodgkin's disease and lymphosarcomas and in the bone marrow aspirates of patients with autoimmune anaemias (2, 13) or transient histiocytosis.

thalassemia syndromes, were examined. 65 of these had Hb H inclusion bodies studies, and 23 had inclusion bodies and globin chain synthesis analysis.

#### Methods

Blood samples were collected in EDTA. Hematological measurements were made with Coulter Model S or ZBI electronic counter standardized daily with commercial standard. For quantitative estimation of individual Hb, the electrophoresis was carried out on Titan III cellulose acetate plate (Helena Laboratories, Beaumont, Tex.). Hb A<sub>2</sub> was quantitated by DE 52 microchromatography [4]. Alkali-resistant Hb was estimated according to the procedure of Fowdery et al. [7]. Osmotic fragility was assessed according to Silverstein and Bianco [8]. Serum iron and iron binding capacity was determined by the method of Leiber [6].

Preparation for detection of Hb H inclusion bodies in erythrocytes was made mixing one part of whole blood with one part of 1% brilliant cresyl blue in 0.9% NaCl and incubating at 37°C for 1 h. Observation of the smears was prolonged to 1 h. Globin chain synthesis analysis was performed according to Kan et al. [5].

## Results

### Globin Chain Synthesis Analysis

Figure 1 shows a chromatogram of a representative case. Figure 2 shows the

$\alpha/\beta$  ratios of the subjects under study. The ratios of normal controls and Sardinian patients with Hb H disease and their parents are also shown for comparison [3 and unpubl. results]. As the mean  $\alpha/\beta$  ratio of the study group was significantly lower than of controls with no overlap, they were considered  $\alpha$ -thal carriers. This ratio ( $0.70 \pm 0.10$ ) is the same as that already observed by us in a group of obligate  $\alpha$ -thal<sup>+</sup> heterozygotes (whose children have Hb H disease). However as shown in figure 2, there is overlap on one hand, with the ratios of Hb H disease patients, and on the other with those of hematologically normal parents of these patients ( $\alpha$ -thal<sup>+</sup>).

### Hb H Inclusion Bodies Studies

Erythrocyte-containing inclusion bodies of  $\alpha$ -thal carriers can be seen in figure 3. On careful examination of blood smears, 74% of subjects under study with the previously defined thalassemia trait showed at least one cell with Hb H inclusion bodies. A similar percentage (65%) was found in subjects defined as  $\alpha$ -thal carriers on globin chain synthesis analysis.

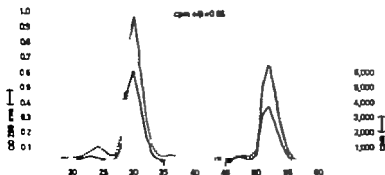


Fig. 1. Radiochromatogram of an  $\alpha$ -thal trait subject.

## Hematological Characteristics of Sardinian $\alpha$ -Thalassemia Carriers Detected in a Population Study

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**Key Words.**  $\alpha$  Thalassemia Globin chain synthesis Hb H inclusion bodies

**Abstract.** 88 adults with thalassemia like red cell indices, normal serum iron and normal hemoglobin (Hb)  $A_2$  and F levels, diagnosed in a mass screening had Hb H inclusion bodies studies (65 subjects) or Hb H inclusion bodies studies and globin chain synthesis analysis (23 subjects). The  $\alpha/\beta$  ratio of  $0.70 \pm 0.10$  was the same as in obligate  $\alpha$ -thalassemia<sup>1</sup> ( $\alpha$ -thal) carriers. Hb H inclusion bodies studies were found to be a reliable test for  $\alpha$ -thal trait identification resulting positive in approximately 70% of suspected carriers. The  $\alpha$ -thal carrier defined by Hb H preparation or by globin chain synthesis had significant reduction in the mean Hb level hematocrit, mean corpuscular hemoglobin and a significant increase in mean red cell count, but there was some overlap with controls.

### Introduction

A high frequency of both  $\alpha$ - and  $\beta$ -thalassemia (thal) has been found in some racial groups such as Sardinian [2] Cypriot [1] and Thai [10]. In these populations, matings between high hemoglobin (Hb)  $A_2$   $\beta$ -thal and thal carriers with normal Hb  $A_2$  and F levels ( $\delta\beta^+$  thal or  $\alpha$ -thal) may frequently occur. While there is a 25% risk of producing  $\beta$ -thal major or intermedia in high Hb  $A_2$   $\beta$ -thal/ $\delta\beta^+$  thal matings,  $\beta$ -thal/ $\alpha$ -thal matings present no such risk. Therefore, the differentiation between  $\delta\beta^+$ -thal and  $\alpha$ -thal seems to be a basic step in the genetic counselling of such couples.

$\alpha$  thal carrier identification relies on Hb H inclusion bodies studies or globin chain

synthesis analysis. The diagnostic value of the former has been found variable in various racial groups [9] while the latter consists a precise identification but it is expensive and troublesome.

This study was undertaken in order to evaluate the diagnostic values of Hb H inclusion bodies analysis and to define the hematological characteristics of ascertained  $\alpha$ -thal carriers, identified in a mass screening in our population.

### Subjects and Methods

#### Subjects

A group of 88 subjects with thal anemia-like red cell indices, normal serum iron, normal Hb  $A_2$  and F levels, detected in a mass screening for

Table 1. Red blood cell indices and  $\alpha/\beta$  ratio in  $\alpha$ -thal heterozygotes

	$\alpha$ -thal carriers by Hb H preparation only (n = 65)				$\alpha$ -thal carriers by biosynthesis (n = 23)				Obligate $\alpha$ -thal <sup>+</sup> carriers (n = 11)				Normal controls (n = 372)			
	mean $\pm$ SD		range		mean $\pm$ SD		range		mean $\pm$ SD		range		mean $\pm$ SD		range	
RBC, $10^{12}/l$	5.9 $\pm$ 0.6	4.4	7.3		5.8 $\pm$ 0.6	4.8	7.0		5.8 $\pm$ 0.7	4.7	7.3		4.9 $\pm$ 0.5	3.9	6.7	
Hb, g/dl	13.1 $\pm$ 1.5	10.0	16.1		13.8 $\pm$ 1.7	10.8	17.3		12.8 $\pm$ 1.9	9.2	14.7		14.9 $\pm$ 1.3	10.5	19.1	
Hct, %	42.2 $\pm$ 5.2	32.7	53.3		41.4 $\pm$ 4.2	31.6	49.2		41.3 $\pm$ 6.1	29.1	50.2		42.9 $\pm$ 3.7	30.1	51.2	
MCV f	71.7 $\pm$ 4.5	60.0	77.0		72.1 $\pm$ 4.7	62.0	78.0		72.0 $\pm$ 4.8	64.0	79.0		88.3 $\pm$ 5.3	77.0	102	
MCH, pg	22.3 $\pm$ 2.1	17.4	27.5		24.0 $\pm$ 2.1	20.0	27.2		22.2 $\pm$ 2.5	19.5	27.6		30.5 $\pm$ 2.1	25.4	35.0	
MCHC, g/dl	31.0 $\pm$ 2.5	24.7	36.1		33.1 $\pm$ 1.9	28.9	35.6		31.1 $\pm$ 3.1	27.2	37.1		34.1 $\pm$ 0.8	32.6	36.0	
Hb A <sub>2</sub> , %	2.2 $\pm$ 0.3	1.2	2.7		2.3 $\pm$ 0.3	1.6	2.8		2.1 $\pm$ 0.3	1.8	2.8		2.5 $\pm$ 0.3	1.5	3.0	
$\beta$ ratio					0.7 $\pm$ 0.1	0.54	0.83		0.7 $\pm$ 0.06	0.6	0.76					

One of the parents of Hb H disease patients

This mean  $\pm$  SD was calculated from the results obtained on 7 subjects.

of subjects examined. Hb A<sub>2</sub> levels were generally lower than normal controls, but there was no significant difference in mean values.

As can be seen in figure 4 there is a slight correlation between the  $\alpha/\beta$  ratios and

MCV [ $r = 0.44$  ( $p < 0.05$ )] These carriers usually had only slight morphological modifications, like hypochromia, microcytosis, anisopoikilocytosis. However in some cases the peripheral blood smear was indistinguishable from that of normal subjects.

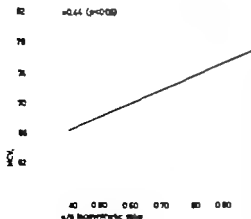


Fig. 4. Correlation between the  $\alpha/\beta$  ratio and MCV in  $\alpha$ -thal trait.  $\circ$  = 1 patient;  $\Delta$  = 2 patients

## Discussion

The result of Hb H inclusion bodies studies showed the diagnostic value of this test in  $\alpha$ -thal carrier identification at least in our population, as has already been seen in Chinese and Greeks [9]. Using this test only 6% undefined thal carriers with normal Hb A<sub>2</sub> and F requires globin chain synthesis studies for definition.

The mean  $\alpha/\beta$  ratios of  $\alpha$ -thal carriers detected in the screening was similar to that of obligate  $\alpha$ -thal carriers in the same population. The overlap with Hb H disease and  $\alpha$ -thal<sup>+</sup> may be due to the failure of the method to differentiate between the differ



### Hematological Analysis

Table I shows hematological indices of defined  $\alpha$ -thal carriers diagnosed on the basis of Hb H inclusion bodies studies or reduced  $\alpha/\beta$  ratios, and obligate  $\alpha$ -thal carriers (one of the parents of Hb H disease patients) As can be seen there is no significant difference between these two groups

The definite  $\alpha$ -thal carriers examined showed a significant reduction in the mean Hb level, hematocrit (Hct) mean corpuscular hemoglobin (MCH) and a significant increase in mean red cell count. However the range of values overlapped with that of controls.

Osmotic fragility was decreased in 71%

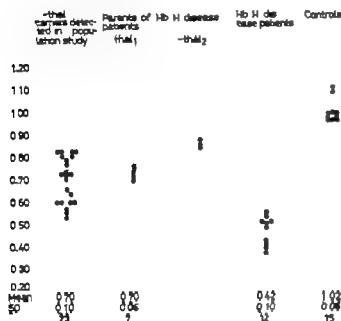


Fig. 2. Results of globin chain synthesis analysis in Hb H disease patients, their parents,  $\alpha$ -thal carriers detected in a screening program and controls.



Fig. 3. Erythrocyte containing Hb H inclusion bodies of an  $\alpha$ -thal trait subject.

Table 1. Red blood cell indices and  $\alpha/\beta$  ratio in  $\alpha$ -thal heterozygotes

	$\alpha$ -thal carriers by Hb H preparation only (n = 65)			$\alpha$ -thal carriers by biosynthesis (n = 23)			Obligate $\alpha$ -thal <sup>-</sup> carriers (n = 11)			Normal controls (n = 372)		
	mean $\pm$ SD range			mean $\pm$ SD range			mean $\pm$ SD range			mean $\pm$ SD range		
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Hb A <sub>2</sub> , %	2.2 $\pm$ 0.3	1.2	2.7	2.3 $\pm$ 0.3	1.6	2.8	2.1 $\pm$ 0.3	1.8	2.8	2.5 $\pm$ 0.3	1.5	3.0
$\beta$ ratio				0.7 $\pm$ 0.1	0.54	0.83	0.7 $\pm$ 0.06 <sup>a</sup>	0.6	0.76			

One of the parents of Hb H disease patients

This mean  $\pm$  SD was calculated from the results obtained in 7 subjects.

of subjects examined. Hb A<sub>2</sub> levels were generally lower than normal controls, but there was no significant difference in mean values.

As can be seen in figure 4 there is a slight correlation between the  $\alpha/\beta$  ratios and

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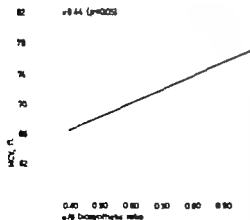


Fig. 4. Correlation between the  $\alpha/\beta$  ratio and MCV in  $\alpha$ -thal trait.  $\circ$  = 1 patient  $\Delta$  = 2 patients.

## Discussion

The result of Hb H inclusion bodies studies showed the diagnostic value of this test in  $\alpha$ -thal carrier identification at least in our population as has already been seen in Chinese and Greeks [9]. Using this test only 26% undefined  $\alpha$ -thal carriers with normal Hb A and F requires globin chain synthesis studies for definition.

The mean  $\alpha/\beta$  ratios of  $\alpha$ -thal carriers detected in the screening was similar to that of obligate  $\alpha$ -thal<sup>-</sup> carriers in the same population. The overlap with Hb H disease and  $\alpha$ -thal may be due to the failure of the method to differentiate between the differ

ent  $\alpha$ -thal genotypes. However it should be considered that some  $\alpha$ -thal<sup>-2</sup> carriers could have microcytosis, the criteria used by us in selecting cases for examination.

Globin chain synthesis analysis in  $\alpha$ -thal heterozygotes of different racial groups showed a considerable degree of heterogeneity. This may be due to variation in technique, different diets or genetic heterogeneity.

The  $\alpha$ -thal carriers showed variable hematological manifestations in different racial groups (Indians, Greeks, Chinese) [9]. Sardinian subjects had mean hematological values similar to those found in Indians and Greeks.

In conclusion this study has practical implications for genetic counselling of high Hb A<sub>2</sub>  $\beta$ -thal/undefined  $\beta$ -thal trait (normal Hb A<sub>2</sub> and F levels) couples. To define the latter and so determine the risk, globin chain synthesis analysis should always be carried out, if Hb H inclusion bodies studies result to be negative.

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## $\beta^0$ Thalassemia Complicated by Autoimmune Hemolytic Anemia

Globin Synthesis during Immunosuppressive Therapy

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**Key Words:** Globin synthesis Immune hemolytic anemia Immunosuppressive therapy  $\beta^0$  Thalassemia

**Abstract.** The unusual occurrence of both  $\beta^0$ -thalassemia and autoimmune hemolytic anemia (AIHA) in a 28-month-old child is reported to illustrate special diagnostic and therapeutic studies. The diagnosis of  $\beta^0$ -thalassemia was established by clinical, family and globin synthesis studies. The diagnosis of AIHA was confirmed by the shortened survival of transfused autologous and homologous red blood cells and by positive direct antiglobulin tests. During treatment of the AIHA with corticosteroids and various immunosuppressive drugs, globin synthesis studies were performed to evaluate the coincidental effects on  $\gamma/\alpha$ -globin chain synthetic ratios. A 50% increase in the  $\gamma/\alpha$  synthetic ratio during cyclophosphamide treatment suggests that further studies of the possible benefits of cytotoxic drug therapy in  $\beta$ -thalassemia may be indicated.

A child with homozygous  $\beta^0$ -thalassemia developed an acute autoimmune hemolytic anemia (AIHA) requiring 94 transfusions of red blood cells during a 16 month period. The coincidence of an inherited and an acquired hemolytic anemia in a young child presented uncommon diagnostic problems, but provided the unusual opportunity for studying the effects of several cytotoxic drugs on the abnormal globin synthesis in  $\beta^0$ -thalassemia.

### Case Report

A 31-month-old Jewish girl of Moroccan extraction was found to have moderate anemia since the age of 1 year. The diagnosis of homozygous  $\beta^0$ -thalassemia was established on the basis of family and hematologic studies (table I) and was later confirmed by radiochromatography of globin chains labeled with  $^{14}\text{C}$ -leucine (fig. 1). The patient did not require blood transfusions until, at the age of 28 months, she suddenly developed jaundice and progressive anemia. The frequency

ent  $\alpha$ -thal genotypes. However it should be considered that some  $\alpha$ -thal<sup>-2</sup> carriers could have microcytosis, the criteria used by us in selecting cases for examination

Globin chain synthesis analysis in  $\alpha$  thal heterozygotes of different racial groups showed a considerable degree of heterogeneity. This may be due to variation in technique, different diets or genetic heterogeneity.

The  $\alpha$ -thal carriers showed variable hematological manifestations in different racial groups (Indians, Greeks, Chinese) [9]. Sardinian subjects had mean hematological values similar to those found in Indians and Greeks.

In conclusion, this study has practical implications for genetic counselling of high Hb A<sub>2</sub>  $\beta$ -thal/undefined thal trait (normal Hb A<sub>2</sub> and F levels) couples. To define the latter and so determine the risk, globin chain synthesis analysis should always be carried out, if Hb H inclusion bodies studies result to be negative.

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### Course and Treatment

The girl received 54 transfusions of red cell concentrates during a 16-month period, and some times required as many as four transfusions per week to maintain hemoglobin level higher than 5-6 g/dl. Treatment with oral prednisone (30 mg/day), cyclophosphamide (50 mg/day) and methotrexate (2.5 mg/day) did not appear to reduce the hemolysis (Fig 2). A transient decrease in her transfusion requirement was observed after initiation of treatment with vincristine (1 mg/week), antihuman lymphocytic globulin (Behringwerke AG Marburg-Lahn, FRG 125 mg/day i.v.) and actinomycin D (15  $\mu$ g/kg/day  $\times$  5 days). Starting from 1 week after the first treatment with actinomycin D, the patient did not require blood transfusions for 28 days, but the beneficial effect was not observed after second course of actinomycin D. No unusual side effects from these drugs were observed. At age 38 months the spleen had enlarged to 18 cm below the costal margin causing abdominal discomfort and splenectomy was performed. Splenectomy also failed to alter the severity of the hemolysis. The child died at the age of 45 months of bronchopneumonia, pericardial effusion and congestive heart failure.

### Special Investigations

#### Antibody Studies

The initial studies at Hadassah Hospital in February 1977 revealed a negative direct antiglobulin test (DAT) both with 'broad spectrum' antihuman serum and with mono-specific anti-IgG. IgA, IgM,  $-C_3$  and  $-C_4$  sera. The patient's blood type was B-negative and her phenotype was *rh(cde/cde) kk*. The indirect antiglobulin test revealed anti-rh (E) and anti-Kell IgG alloantibodies, both of which were detectable for only a few weeks duration. There was also a transient IgM anti-I which had a low titer at 4 C (32) and was not detectable above 22 C. After approximately 30 red cell transfusions, the patient developed a fourth allo-

antibody anti-rh<sup>-1</sup> (C<sup>+</sup>), which persisted throughout her hospitalization.

Immediately after splenectomy the DAT became positive (2+) with both 'broad spectrum' antihuman serum and with mono-specific anti-IgG serum (2+). Acid and heat eluates of the autoantibody revealed a panagglutinin with no detectable specificity for Rh or other red cell phenotypes.

#### RBC Survival

These studies were performed before the child underwent splenectomy. Survival of compatible red cells obtained from a normal newborn child, and labelled with  $^{51}\text{Cr}$  was extremely shortened ( $T_{1/2}$  = 36 h). Red cells from a newborn were selected because of their low content of I antigen. However  $^{51}\text{Cr}$  and other techniques for mixed cell labelling would have been inadequate for the labelling of autologous cells, since the child required red cell transfusions every few days. Therefore, cohort labelling of reticulocytes was performed [1] using 50 ml of the patient's heparinized venous blood drawn in sterile plastic transfer packs (Fenwall Labs., TA 2) and incubated for 2 h at 37 C with 50  $\mu\text{Ci}$   $^{14}\text{C}$  leucine (New England Nuclear Boston, Mass., 0.3 Ci/mM). Separately 350 ml of compatible heparinized homologous whole blood was incubated with 1 mCi  $^3\text{H}$ -leucine (New England Nuclear 30 Ci/mM). Excess isotope was removed from each transfer pack by three washings, with 0.9% sodium chloride. The labelled autologous and homologous red cells were combined in a disposable cell wash bowl (Haemonetics Corp. No. 5510) and washed automatically (Haemonetics Model 15 Cell Washer) with 2 liters of buffered 0.2% dextrose and 0.8% sodium chloride. The washed labelled red cells were resuspended

of red cell transfusions required to maintain her hemoglobin level above 6 g/dl increased to more than one per week, and she was transferred from the Barzilai Medical Center to the Hadassah Hospital for further studies.

On admission she was pale, jaundiced and dyspneic. She had the typical facies of thalassemia major and was underdeveloped for her age. The abdomen was protruding, with the spleen palpable 12 cm, and liver 4 cm, below the costal margin. A systolic murmur was present over the entire precordium.

#### Laboratory Results

There was a normocytic normochromic anemia, with a hemoglobin of 5.2 g/dl and 3% reticu-

locytes. Leukocytes and platelet counts were only slightly elevated. The peripheral blood smear revealed occasional normoblasts, spherocytes and rouleaux formation. The direct antiglobulin test (DAT) was negative. Smears of the bone marrow aspirate showed marked erythroid hyperplasia. The serum was clear with a red-brown color. Serum chemistry examinations, performed a few days after the last blood transfusion, revealed bilirubinemia (4.5 mg/dl) and hyperuricemia (8.1 mg/dl). Kidney and liver function tests were normal. Hemoglobin was 15 mg/dl and  $C_3$  complement was 60 mg/dl. The acid serum test (Ham) was negative. There was gross hemoglobinuria, particularly after blood transfusions, reaching as high as 37 g/dl on one occasion.

Table I. Hemoglobin studies in the patient and family

Subject	Age years	Hemoglobin concentration, g/dl	Hemoglobin electrophoresis		
			hemoglobin A	hemoglobin F %	hemoglobin A <sub>2</sub> %
Patient	16/12	8.2 <sup>1</sup>	absent	81.0	1.8
Father	41	12.2	major component	1.3	5.5
Mother	35	12.0	major component	2.4	5.2
Sister	15	10.9	major component	1.0	5.4
Brother	10	10.8	major component	1.2	6.2
Sister	6	9.3	major component	2.0	6.6

Prior to AIHA and transfusions.

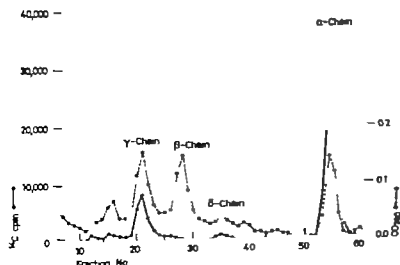


Fig. 1. CM cellulose radiochromatography of globin chains in bone marrow cells of the patient (Feb. 77).

### *Course and Treatment*

The girl received 94 transfusions of red cell concentrates during a 16-month period, and some times required as many as four transfusions per week to maintain hemoglobin level higher than 5.6 g/dl. Treatment with oral prednisone (30 mg/day), cyclophosphamide (50 mg/day) and methotrexate (2.5 mg/day) did not appear to reduce the hemolysis (fig. 2). A transient decrease in her transfusion requirement was observed after initiation of treatment with vincristine (1 mg/week), asthromax lymphocytic globulin (Behringwerke AG Marburg-Lahn, FRG 125 mg/day i.v.) and actinomycin D (15  $\mu$ g/kg/day  $\times$  5 days). Starting from 1 week after the first treatment with actinomycin D, the patient did not require blood transfusions for 24 days, but the beneficial effect was not observed after second course of actinomycin D. No unusual side effects from these drugs were observed. At age 38 months the spleen had enlarged to 18 cm below the costal margin causing abdominal discomfort and splenectomy was performed. Splenectomy also failed to alter the severity of the hemolysis. The child died at the age of 43 months of bronchopneumonia, pericardial effusion and congestive heart failure.

### *Special Investigations*

#### *Antibody Studies*

The initial studies at Hadassah Hospital in February 1977 revealed a negative direct antiglobulin test (DAT) both with 'broad spectrum' antihuman serum and with mono-specific anti-IgG -IgA, -IgM, -C<sub>3</sub> and -C<sub>4</sub> sera. The patient's blood type was B-negative and her phenotype was rh(cde/cde) kk. The indirect antiglobulin test revealed anti-rh (E) and anti-Kell IgG alloantibodies, both of which were detectable for only a few weeks duration. There was also a transient IgM anti-I which had a low titer at 4°C (32) and was not detectable above 22°C. After approximately 30 red cell transfusions, the patient developed a fourth allo-

antibody anti-rh<sup>41</sup> (C<sup>a</sup>), which persisted throughout her hospitalization.

Immediately after splenectomy the DAT became positive (2+) with both 'broad spectrum' antihuman serum and with mono-specific anti-IgG serum (2+). Acid and heat eluates of the autoantibody revealed a panagglutinin with no detectable specificity for Rh or other red cell phenotypes.

#### *RBC Survival*

These studies were performed before the child underwent splenectomy. Survival of compatible red cells obtained from a normal newborn child, and labelled with <sup>51</sup>Cr was extremely shortened (T<sub>1/2</sub> = 36 h). Red cells from a newborn were selected because of their low content of I antigen. However <sup>51</sup>Cr and other techniques for mixed cell labelling would have been inadequate for the labelling of autologous cells, since the child required red cell transfusions every few days. Therefore, cohort labelling of reticulocytes was performed [1] using 50 ml of the patient's heparinized venous blood drawn in sterile plastic transfer packs (Fenwall Labs., TA 2) and incubated for 2 h at 37°C with 50  $\mu$ Cl <sup>14</sup>C leucine (New England Nuclear Boston, Mass., 0.3 Ci/mM). Separately 350 ml of compatible heparinized homologous whole blood was incubated with 1 mCi <sup>3</sup>H-leucine (New England Nuclear 30 Ci/mM). Excess isotope was removed from each transfer pack by three washings, with 0.9% sodium chloride. The labelled autologous and homologous red cells were combined in a disposable cell wash bowl (Haemonetics Corp. No 5510) and washed automatically (Haemonetics Model 15 Cell Washer) with 2 liters of buffered 0.2% dextrose and 0.8% sodium chloride. The washed labelled red cells were resuspended



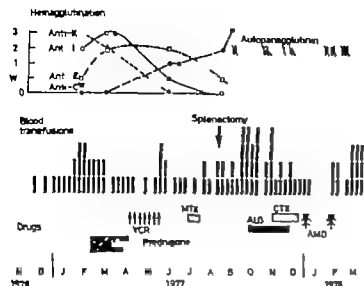


Fig. 2. Diagrammatic representation of the course of the disease over a period of 17 months. Each blood transfusion is represented by a block. The strength of hemagglutination with various antisera is represented in the upper panel. Administered drugs are represented below the blood transfusions. VCR = Vincristine; MTX = methotrexate; ALG = antilymphocytic globulin, AMD = actinomycin D CTX = cyclophosphamide.

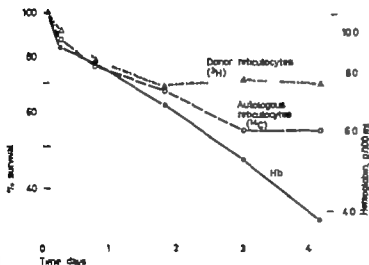


Fig. 3. Rapid drop of hemoglobin level (●) and percent survival of  $^{14}\text{C}$  labelled autologous reticulocytes (○) and of  $^3\text{H}$ -labelled homologous reticulocytes (Δ) after their intravenous infusion.

in this solution and transfused as packed cells. The injected radioactivity was  $61 \mu\text{Ci } ^3\text{H}$  and  $2.0 \mu\text{Ci } ^{14}\text{C}$ . Heparinized blood samples were withdrawn at various time intervals after the infusion of labelled red cells for determination of  $^3\text{H}$  and  $^{14}\text{C}$  red cells survivals, as well as for hemoglobin and hematocrit determinations. Because of the high quenching of whole red cell hemolysate, globin was extracted from the var-

ious samples with acid acetone and dissolved in  $0.2 \text{ N NaOH}$ . An aliquot of  $1.5 \text{ ml}$  from each globin sample, containing  $50\text{--}82 \text{ mg}$  globin, was counted in duplicate in  $17 \text{ ml}$  Aquasol (New England Nuclear). In each sample  $^3\text{H}$  radioactivity was higher than  $2,000 \text{ cpm}$  and  $^{14}\text{C}$  radioactivity was higher than  $250 \text{ cpm}$ . Correction for quenching and spilling was based on internal  $^3\text{H}$  and  $^{14}\text{C}$  standards.  $^3\text{H}$  and  $^{14}\text{C}$  ra-

Table II. Globin synthetic ratios during treatment with various cytotoxic drugs

Date	Treatment (dosage)	Peripheral blood		Bone marrow	
		$\gamma/\delta$	$\delta/\alpha$	$\gamma/\alpha$	$\delta$
Feb. 2, 77	prednisone (30 mg/day per os)	0.29	0	0.21	0.02
Apr. 17, 77	prednisone (20 mg/day per os)	0.29	< 0.01	—	—
Apr. 23, 77	vincristine (1 week after 1 mg i. v.)	0.31	0	0.21	0.01
May 16, 77	vincristine (1 week after 1 mg i. v.)	0.27	0	0.22	0.02
July 25, 77	methotrexate (2.5 mg/day per os)	0.28	0	0.19	0.02
Dec. 12, 77	cyclophosphamide (30 mg/day per os)	0.45	< 0.01	—	—
Dec. 19, 77	cyclophosphamide (30 mg/day per os)	0.48	0	0.28	0.02
Dec. 27, 77	none	0.32	0	0.24	0.03
Feb. 2, 78	actinomycin D (0.18 mg/day i. v.)	not enough incorporation		0.19	0.02

radioactivity per gram globin at each time interval was multiplied by the hematocrit and divided by the radioactivity  $\times$  hematocrit factor 2 h after transfusion of the labelled red cells, to determine the percentage survival of labelled reticulocytes. The results of this experiment are presented in figure 3. Rapid destruction of autologous as well as homologous reticulocytes within just a few days is evident.

### Globin Synthesis

Globin was prepared from peripheral blood and bone marrow cells labelled *in vitro* with  $^3\text{H}$ -leucine and analyzed by urea carboxymethyl cellulose chromatography with methods previously described [2]. The study was repeated on several occasions to explore the possible beneficial effects of various cytotoxic drugs on globin synthetic ratios. No synthesis of  $\beta$ -chains was ever detected (fig. 1). Delta chains were synthesized mainly in bone marrow cells. A relatively high  $\gamma/\delta$  synthetic ratio (0.20) was measured in bone marrow cells. The ratio was fairly constant throughout the course of the disease and was neither affected by the hemoglobin level, frequency of transfusions, nor

treatment with prednisone, vincristine, methotrexate, antilymphocytic globulin or actinomycin D. During treatment with cyclophosphamide an increase in  $\gamma/\alpha$  synthetic ratios of about 50% was observed in both peripheral blood and bone marrow cells (table II).

### Discussion

The clinical and laboratory findings in the present case represent a relatively mild course of  $\beta^0$ -thalassemia complicated by AIHA. The occurrence of both an inherited and an acquired hemolytic anemia in a young child is intriguing, but probably coincidental. What is unique in this case, however, was the opportunity for observing the effects of immunosuppressive therapy with several different cytotoxic drugs on the abnormal globin synthesis in  $\beta^0$ -thalassemia.

The diagnosis of AIHA was not immediately apparent, because the DAT was repeatedly negative prior to splenectomy. However, AIHA was suggested by the appearance of spherocytes and rouleaux formation in the peripheral blood smear and

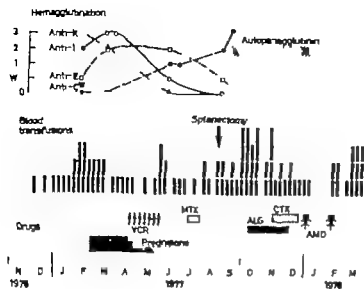


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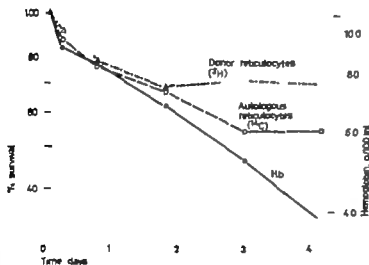


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vestigation of cyclophosphamide treatment in patients with  $\beta$ -thalassaemia.

### Acknowledgements

This work was supported by grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel.

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by the rapid destruction of  $^{14}\text{C}$  labelled autologous as well as  $^3\text{H}$  labelled homologous red cells. While a small percentage of patients with AIHA have negative DAT probably because of the low number of immunoglobulin molecules coating the red cells [3] the present case appears to represent an alternative mechanism. The temporal relationship of the patient's conversion from negative to positive DAT after splenectomy suggests that immunoglobulin-coated red cells were sequestered and destroyed upon passage through her massively enlarged spleen and that sufficient numbers of immunoglobulin-coated red cells were not released into the circulation for detection until after splenectomy. An analogous situation may occur in hereditary spherocytosis, where the percentage of spherocytes in the peripheral blood increases after splenectomy [4] or in thalassemia, where the number of inclusion bodies also increases after splenectomy [5]. Accordingly we suggest that the coincidental presence of an enlarged spleen be considered among the causes of negative DAT in AIHA.

The decision for treatment with immunosuppressive drugs was based upon the failure to control her hemolytic disease by corticosteroids, the increasing transfusion requirement, and the increasing difficulty of preparing red cell transfusions compatible with her multiple alloantibodies. The results of different approaches to the use of cytotoxic drugs in patients with AIHA have been summarized recently [6]. In our patient, treatment with prednisone, methotrexate, antilymphocytic globulin and cyclophosphamide were not beneficial, and treatment with vincristine and actinomycin D were only transiently effective in reducing the transfusion requirement. Some of these

drugs are not used commonly in autoimmune disorders but were tried when everything else failed. The dose used was not sufficient to reduce the leukocyte counts to below  $5\,000/\text{mm}^3$ .

During treatment with the various cytotoxic drugs, studies were performed to observe the coincidental effects on the patient's abnormal globin synthesis (table II). Of the several drugs evaluated, only cyclophosphamide had an observed effect in ameliorating the  $\alpha$  versus  $\gamma$  chain imbalance, and this effect was observed in peripheral blood on two separate occasions and once in bone marrow cells. The mechanism by which cyclophosphamide alters globin synthesis in thalassemia is not known, but it is possible that cyclophosphamide preferentially inhibits  $\alpha$  chain synthesis. If this is the case cyclophosphamide should also increase  $\beta/\alpha$  synthetic ratios in patients with homozygous  $\beta^+$ -thalassemia. Alternatively the observed effect may be related to a preferential survival of fetal hemoglobin producing cells, analogous to the situation when hereditary persistence of fetal hemoglobin interacts with  $\beta$ -thalassemia [7].

Recently 3 patients with homozygous  $\beta^0$  thalassemia were reported who did not require blood transfusions [8]. Their comparatively mild clinical courses were attributed to  $\gamma/\alpha$  synthetic ratios that were higher than those usually reported in this disorder. If increases in  $\gamma/\alpha$  synthetic ratios such as those observed in our patient during cyclophosphamide therapy could be reproduced in other patients with  $\beta$ -thalassemia, it is feasible that clinically significant benefits might be achieved. This objective, combined with the anticipated suppression of bone marrow hyperplasia and extramedullary hematopoiesis, may justify further in

vestigation of cyclophosphamide treatment in patients with  $\beta$ -thalassemia.

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## Thalassemia in Southern India

### Interaction of Genes for $\beta^+$ $\beta$ , and $\delta^0\beta^0$ Thalassemia

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Divisions of Hematology and Geographic Medicine, Departments of Medicine and Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio; Department of Pediatrics, Holdsworth Memorial Hospital, Mysore City, Karnataka, India, and Laboratory of Protein Chemistry and Comprehensive Sickle Cell Center, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Ga.

**Key Words.**  $\beta$ -Thalassemia genes  $\beta$  Thalassemia intermedia

**Abstract.** Two families from southern India with members having the clinical manifestations of thalassemia intermedia are presented. Hematologic and globin chain synthesis data indicated that in one family the affected brother and sister had the  $\beta^+-\delta$   $\beta^0$  thalassemia condition while in the other family the proband was homozygous for a  $\beta^0$ -thalassemia determinant.

The diversity of the forms of  $\beta$ -thalassemia in India is not widely appreciated. Although the thalassemia syndromes are the most common hemoglobinopathies in India [1, 2] and hundreds of patients with thalassemia major have been recognized [3], most were reported before the genetic complexity underlying this condition was apparent. Recently 3 individuals from two southern Indian families with the clinical manifestations of thalassemia intermedia were referred to one of us (VH). Subsequent studies showed that genes for  $\beta^+$   $\beta$  and  $\delta$   $\beta^0$  thalassemia were segregating in these families.

### Materials and Methods

19 members of two families were studied, both had members who had been referred to Hold-

worth Memorial Hospital, Mysore, Karnataka for consultation. After obtaining informed consent, medical histories and pedigrees, a physical examination was performed and blood samples were collected by venipuncture in EDTA Vacutainer tubes.

#### *Studies in India*

**Hematologic Methods.** Within 12 h the PCV value was determined by microhematocrit with an International Equipment Company centrifuge, the Hb level was measured as cyanomethHb using a Bausch and Lomb Spectronic mini-20 spectrophotometer and manual erythrocyte counts were made using a Nikon H3 microscope [4]. Coverlip blood smears were prepared and fixed in methanol for subsequent Wright's stain [4] and an acid elution stain for the distribution of Hb F was done [5]. Plasma was then frozen, erythrocytes refrigerated at 4 °C and both transported to the US by air within 21 days.

#### *Studies in the United States*

Plasma was carried to Cleveland, Ohio where plasma folate [6], B<sub>12</sub> [7], and iron levels [8] were

measured in all specimens; in some instances plasma ferritin was determined using kit method (Rusco Laboratories, Inc.). Erythrocytes were carried to Augusta, Ga. for hemoglobin analysis (for technical details see *Huisman and Jonxis* [9]). All samples were studied by starch-gel electrophoresis while percentages of Hb A, Hb F and Hb A<sub>2</sub> were determined by DEAE-cellulose column chromatography. Hb F was also quantitated by an alkal denaturation and Hb A<sub>2</sub> by microcolumn chromatography.

**Globin-Chain Synthesis.** Blood samples were collected in EDTA anticoagulant and processed immediately in India, using centrifugation method to concentrate reticulocytes. After incubation with [<sup>14</sup>C]-leucine for 2 h at 37 °C, cells were lysed with distilled water, frozen and transported by air to Augusta, Ga. Whole cell globin was used in the final analysis.

## Results and Interpretations

Pedigrees of the families are shown in figure 1 and clinical and hematological data in table I.

### Family CA

This Hindu family was from the Coorg district of Karnataka. The proposita (CA III 1) was referred for evaluation of intermittent jaundice. Both she and her younger brother (CA III 2) had been otherwise well throughout their lives, with normal growth and development. Neither had ever received a blood transfusion. Examination revealed no cephalofacial deformity but both had splenomegaly. Stained peripheral blood films from both showed moderate to severe microcytosis and hypochromia with anisopoikilocytosis, polychromatophilia, and an occasional target cell. The father and his siblings all had elevated Hb A<sub>2</sub> values with normal levels of Hb F while the mother and her siblings had normal Hb A<sub>2</sub> levels but with Hb F levels of 5–11 %. This Hb F was

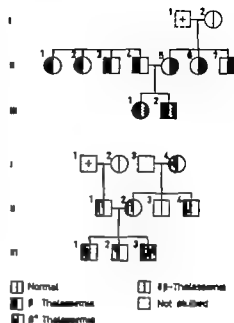


Fig. 1. Pedigrees of families CA (top) and PA (bottom).

heterogeneously distributed among the red cells. The  $\alpha/\text{non-}\alpha$  biosynthetic ratios were 1.25 in the mother, 3.23 in the daughter and 2.94 in the son. About 5% of Hb A was present in both son and daughter. These data and the hematological observations indicate that the father and his siblings are heterozygous for a  $\beta^+$ -thalassemia gene while the mother and her siblings are heterozygous for a  $\delta$   $\beta^0$ -thalassemia gene. Thus, the son and daughter have the  $\beta^+-\delta$   $\beta^0$  thalassemia condition.

### Family PA

This Hindu family was from the Mandya district of Karnataka. The propositus (PA III 3), 5 years of age, was referred because of anemia and hepatosplenomegaly. Pallor had first been noticed when he was about 6



## Thalassemia in Southern India

### Interaction of Genes for $\beta^+$ $\beta$ and $\delta\beta^0$ Thalassemia

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#### *Studies in the United States*

Plasma was carried to Cleveland, Ohio where plasma folate [6], B [7], and iron levels [8] were

abnormalities, slight to moderate hepatosplenomegaly mild anemia and absent transfusion requirements. The clinical expression of the  $\beta^+$ - $\delta$   $\beta^0$ -thalassemia condition in CA III 1 and 2 as thalassemia intermedia is in accord with the typical findings elsewhere the results of the hematologic studies, quantitation of hemoglobin fractions and globin synthesis studies in family CA are all in the ranges reported for other ethnic groups [10-13]

In contrast, the mild clinical course in PA III 3 is unexpected because homozygous  $\beta^0$ -thalassemia is usually a severe disorder requiring frequent blood transfusions from childhood [14]

In India, two individuals homozygous for  $\beta^0$ -thalassemia have been reported by *Sukumaran et al.* [15] one was also heterozygous for Hb Q but both had severe thalassemia major manifestations. Homozygous  $\beta$ -thalassemia intermedia has been described previously but it now appears that a gene for the heterocellular form of the hereditary persistence of fetal hemoglobin [16] might explain the findings both in the Dutch family reported by *Schokker et al.* [17] and the US family described in the abstract by *McElroy et al.* [18] In family PA, however there was no elevation of the Hb F level above that usually seen in  $\beta^0$ -thalassemia heterozygotes to suggest this possibility. The propositus (PA III 3) thus resembles the three Algerian patients with homozygous  $\beta$ -thalassemia intermedia recently described by *Chédali et al.* [19]

The investigations described here permitted identification of  $\beta^+$ ,  $\beta^0$  and  $\delta$   $\beta$ -thalassemia genes segregating in these southern Indian families. Each of these types of thalassemia is now known to be in turn heterogeneous at the molecular level,

so with more detailed analysis further differences between the thalassemias in India and those found elsewhere are likely to become evident. In this regard, the unusually mild course of homozygous  $\beta^0$ -thalassemia in Family PA is particularly noteworthy. Thalassemia is much more common in northern Indian populations and hundreds of cases of thalassemia major have been recognized in the major hospitals of New Delhi, Bombay and Calcutta [3]. Detailed characterizations of the defects underlying the thalassemia syndromes in India would undoubtedly make a great contribution to the understanding of both the clinical expression and the general molecular basis of these disorders.

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Table 1. Hematologic and globin synthesis data in families with  $\beta$ -thalassaemia

Subject	Age and sex	Hb g/dl	PCV l/l	RBC $10^{12}/l$	MCV fl	MCH pg	MCHC g/dl	Reticulocytes %	HbA %	HbA <sub>1</sub> %	HbF %
Family CA											
I 2	69F	14.2	0.44	6.1	72	23	32	—	96.8	2.5	0.7
II 1	63F	11.7	0.42	5.3	79	22	28	—	95.4	4.0	0.6
II 2	56F	12.6	0.44	6.5	68	19	29	—	95.4	4.0	0.6
II 3	41M	14.2	0.46	6.7	68	21	31	—	94.7	4.6	0.7
II 4	48M	13.8	0.45	6.6	68	21	31	0.8	95.0	4.4	0.6
II 5	40F	14.3	0.41	5.5	73	26	35	1.4	88.3	2.4	9.3
II 6	34F	13.2	0.43	5.3	81	25	31	—	91.4	3.0	3.6
II 7	38M	14.4	0.48	5.8	81	25	30	—	91.7	2.5	3.8
III 1 <sup>1</sup>	13F	10.9	0.35	5.4	65	20	31	5.2	5.2	1.8	92.8
III 2 <sup>1</sup>	12M	11.8	0.34	4.8	71	25	35	5.6	5.4	2.3	92.3
Family PA											
I 2	76F	13.6	0.40	4.7	86	29	34	—	96.5	3.2	0.3
I 4	60F	10.5	0.37	6.1	61	17	28	—	93.9	5.4	0.7
II 1	42M	13.7	0.47	6.5	73	21	29	1.0	93.2	5.2	1.6
II 2	31F	13.7	0.45	6.4	70	21	30	1.2	93.9	5.0	1.1
II 3	21M	15.9	0.47	4.9	94	32	34	—	97.1	2.6	0.3
II 4	19M	12.8	0.45	7.1	61	18	29	—	94.1	5.3	0.6
III 1	14M	12.3	0.39	6.1	64	20	32	—	98.0	5.1	<1.0
III 2	8M	12.7	0.40	5.9	68	22	33	—	90.8	6.5	2.7
III 3 <sup>1</sup>	5M	9.8	0.28	3.7	76	26	35	2.5	0	2.3	97.7

HbA<sub>1</sub> determined by microcolumn chromatography and HbF by alkali denaturation.

<sup>1</sup> Plasma iron, ferritin and folate values were within the normal range; the plasma ferritin values were  $\geq 340 \mu\text{g/l}$ .

months old but he had never received a blood transfusion. On examination mild cephalofacial deformity, slight growth impairment, and moderate hepatosplenomegaly were present. The stained peripheral blood film showed marked hypochromic microcytosis with anisopoikilocytosis, target cells and some basophilic stippling. Electrophoretic and chromatographic examination of the hemoglobin indicated the presence of Hb A<sub>2</sub> and Hb F only. Elevated Hb A<sub>2</sub> levels were detected in the siblings, father, mother, a maternal uncle and the maternal grandmother while Hb F levels were normal

or slightly raised. Globin synthesis studies were successful only in the mother (PA II 2); the  $\alpha/\text{non-}\alpha$  ratio was 1.59. Considering all of these data, the proband (PA III 3) is thus homozygous for a  $\beta$ -thalassaemia determinant.

### Discussion

The affected individuals (CA III 1 and 2, PA III 3) in both families have a thalassaemic syndrome of intermediate severity with mild clinical manifestations and bony

# Non-Hodgkin Lymphoma Associated with Double Monoclonal Immunoglobulin

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**Key Words.** Immunoglobulins Lymphomas (non-Hodgkin lymphomas)  
Multiple myeloma

**Abstract.** We describe an unusual case displaying the features of double monoclonal gammopathy (IgM-kappa plus IgG-lambda) associated with non-Hodgkin lymphoma (NHL). In the last years monoclonal gammopathies have been sometimes found to be associated with NHLs. It is a very peculiar occurrence the association between a different class double monoclonal gammopathy and NHL, as the case reported in this paper.

Double monoclonal gammopathies have been frequently described in multiple myeloma. Among other B cell-derived malignant diseases several authors have reported the coexistence of monoclonal gammopathies with non-Hodgkin lymphomas (NHL) but, most frequently the M component was single and belonging to the IgM or more rarely IgG class [1, 2, 4, 5, 8].

In this paper we describe the uncommon case of a NHL associated with a double monoclonal immunoglobulin (IgG-lambda and IgM kappas).

## Case Report

A 73-year-old patient, E. F. was first observed by us 1 month before his death. In 1957 an inguinal lymph node enlargement was observed; at

biopsy lymphosarcoma was diagnosed. He was locally irradiated on regions adjacent to known disease where additional lymphomas had developed during period of about 5 years. He was well until July 1978 when his clinical condition deteriorated suddenly.

A second inguinal lymph node biopsy confirmed the previous diagnosis and bone marrow aspirate study (sternum) was within normal range. A further X-ray treatment of up to 1,600 R was started on an "inverted Y" field below the diaphragm.

Subsequently he was admitted to our Hospital with severe hypochromic anemia, high ESR (95 mm/h), sphincteric disturbances and neurological involvement of both legs. Serum immunoglobulin quantitation according to Mancini revealed: IgG 4,600 mg/100 ml, IgM 1,500 mg/100 ml, IgA 60 mg/100 ml. Serum zone electrophoresis showed two different homogeneous bands in the gammaglobulin region. Serum immunoelectrophoretic analysis by Scheidegger's method allowed us to find gamma and lambda abnormal precipitation

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Fig. 1 Immunoelectrophoresis of the fraction No 31 (indicated by the asterisk) belonging to the first peak eluted from serum gel chromatography on a Sephadex G 200 column. N = Normal serum as control.

arcs showing the same migration features, and a  $\lambda$ -chain irregular precipitation line. Urine analysis revealed an abnormal lambda L-chain precipitation arc quite identical to that found in the serum. Serum gel chromatography showed that the first peak contained an IgM kappa M component. The second peak contained the IgG-lambda monoclonal component as well as normal proteins (fig. 1). The clinical course and second biopsy allowed us to exclude the occurrence of a secondary myeloma. Due to the sudden death of the patient we were unable to continue our investigations.

## Discussion

In recent years, immunological studies on patients suffering from lymphoproliferative diseases emphasized the possible asso-

ciation between monoclonal gammopathies and chronic lymphatic leukemia (CLL) or NHL. The most extensive report has been made by Alexanian [1] who found 45 single-class M components (prevalently IgM) in 1 150 patients including NHL, CLL and Hodgkin lymphomas: a double M component was never observed. Other investigators, on the other hand did not find any monoclonal protein when examining 132 lymphoproliferative malignancies [7].

Lymphoproliferative disorders of the lungs have been described to be associated with serum M components Poirier *et al.* [5] Montes *et al.* [4] and Lavandier *et al.* [2] reported three different cases presenting a monoclonal IgG whereas Ward *et al.* [8] studied a case of an IgM kappa M component associated with pulmonary lymphosarcoma.

Recently Lemenager *et al.* [3] reported a case of primary NHL of the lung which presented a double serum M component belonging to the same (IgM) class.

We report here on an unusual case which displays the features of different-class double monoclonal gammopathy associated with NHL.

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**HLA BW21** The phenotypical frequency of these antigens, compared with the control group was not significantly different (table I). The results obtained might be influenced by the small number of patients studied compared to the number of inhabitants of our country (approximately 10 millions). Although no statistically significant increase for any of the 26 HLA antigens was found, increase in the phenotypical frequency

Table I. Frequencies of 26 specificities among 109 normal Cuban controls and 10 paroxysmal nocturnal hemoglobinuria

Antigen HLA	Normal population 109 controls		Paroxysmal nocturnal hemoglobinuria 10 patients		$\chi^2$ with Yates correction
	positive cases	phenotypic frequency	positive cases	phenotypic frequency	
<i>Locus A</i>					
HLA-A1	14	0.12	1	0.10	0.056 NS
HLA-A2	46	0.42	6	0.60	0.566 NS
HLA-A3	18	0.16	2	0.20	0.025 NS
HLA-A9	16	0.14	3	0.30	0.864 NS
HLA-A10	12	0.11	2	0.20	0.110 NS
HLA-A11	15	0.13	2	0.20	0.004 NS
HLA-A28	9	0.08	1	0.10	0.000 NS
HLA-A29	20	0.18	0	0.00	0.000 NS
HLA-AW30	5	0.04	0	0.00	0.000 NS
HLA-AW33	15	0.13		0.10	0.093 NS
X	31	0.28	1	0.10	NS
<i>Locus B</i>					
HLA-B5	17	0.14	1	0.10	0.000 NS
HLA-B7	18	0.17	5	0.50	4.181 NS
HLA-B8	9	0.08	0	0.00	0.000 NS
HLA-B12	27	0.24	1	0.10	0.441 NS
HLA-B13	6	0.05	0	0.00	0.000 NS
HLA-B14	17	0.15	1	0.10	0.000 NS
HLA-B18	11	0.10	1	0.10	0.000 NS
HLA-B27	6	0.05	1	0.10	0.015 NS
HLA-BW15	6	0.05	1	0.10	0.015 NS
HLA-BW16	5	0.04	0	0.00	0.000 NS
HLA-BW17	13	0.11	0	0.00	0.000 NS
HLA-BW21	6	0.05	3	0.30	4.748 NS
HLA-BW22	5	0.04	1	0.10	0.000 NS
HLA-BW35	31	0.29	2	0.20	0.040 NS
HLA-BW37	5	0.04	0	0.00	0.000 NS
HLA-BW40	7	0.06	1	0.10	0.062 NS
Y	25	0.22	2	0.20	NS

Significant  $p < 0.002$ .



## HLA Antigens in Paroxysmal Nocturnal Hemoglobinuria

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**Key Words.** HLA antigens PNH

**Abstract.** 10 patients with paroxysmal nocturnal hemoglobinuria were studied taking 109 normal subjects of the Cuban population as control group. 26 HLA antigens corresponding to loci A and B were studied in both groups. Phenotypical frequency of both groups were compared. No statistically significant increase was found for any of the studied antigens, though there was a nonsignificant increase for antigens HLA B7 and BW 21. These results might be influenced by the small number of patients studied due to the rareness of the disease.

Although the most important use of the major system of histocompatibility HLA in current medical practice is for organ and tissue transplantation there are now reports of HLA being associated with malignant and nonmalignant disease [1-4].

The purpose of this communication is to report the HLA typing of 10 patients with paroxysmal nocturnal hemoglobinuria (PNH). As far as we know the HLA antigen frequency has not been previously studied in PNH. This has encouraged us to present our results.

The control group was made up of 109 normal subjects living in the City of Havana, studied at the International Histocompatibility Workshop held in Cuba in May 1975. For this work, 117 antisera were used, which determined the existence of 26 antigens, 10 corresponding to loci A and 16 to B. Antigens HLA A1, A2, A3, A9, A10, A11, A28, A29, AW 30 and AW33 were typed from the first group and from the second antigens HLA B5, B7, B8, B12, B13, B14, B18, B27, BW15, BW16, BW17, BW21, BW22, BW35, BW37, BW40. The standardized technique of NIH was used for study of the control group and patients. For statistical analysis the  $\chi^2$  with Yates correction was used.

### Patients and Methods

10 patients with PNH, diagnosed at the Institute of Hematology and Immunology were stud-

### Results and Discussion

It was found in our study that 50% of the patients were HLA B7 and 30% were

## Sézary Syndrome Immunocytological and Cytochemical Variability of Sézary Cells

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**Key Words.** Acid phosphatase tartrate-resistant B cells Null cells Sézary cells Sézary syndrome T cells

**Abstract.** The characteristic large cells in the blood of a patient with Sézary syndrome underwent immunocytological and cytochemical changes during polychemotherapy which caused transient regression of skin lesions. Tartrate-resistant acid phosphatase was demonstrable in a few cells only after chemotherapy initially only 2% T cells and 26% B cells could be demonstrated in the blood by immunocytological methods after cytostatic therapy 35% T cells but no B cells were detected.

### Case Report

We report the case of a patient with histologically haematologically and ultrastructurally confirmed Sézary syndrome, whose atypical cells underwent cytochemical and immunocytological changes during polychemotherapy. The 63-year-old man first noted scaling erythema on his head, shoulder hands and feet in 1975; this was interpreted dermatologically as allergy to mycotic infection and consequently treated with griseofulvin. A smear of aternal marrow at that time showed slight relative increase in lymphoblastic and plasma cells of moderately reduced total cellularity. Skin lesions rapidly progressed early in 1977 and essentially involved the entire integument. The white blood cell count then revealed leucocytosis of 72,000 with 97% lymphocytes, up to 90% of which were typical, and suggested the diagnosis of Sézary syndrome. There was no anaemia or thrombocytopenia (haemoglobin 13.6 g/100 ml, platelets 225,000/mm<sup>3</sup>). Histological and

ultrastructural studies demonstrated typical Sézary cells in the blood (fig 1). Bone marrow biopsy showed no lymphoproliferative infiltration. Histochemically the atypical lymphoid blood cells were predominantly PAS-negative, yet acid-phosphatase (non-tartrate-resistant)- and  $\beta$ -glucuronidase-positive. There were no lymphomas; hepatosplenomegaly was not detected by clinical investigation and scanning methods. Electrophoresis and immunoelectrophoresis of serum proteins were normal.

The immunological T and B cell determination [E, EAC rosettes and immunofluorescence for SIg B] of peripheral blood lymphocytes revealed 2% E rosettes, without cytologically atypical lymphocytes (as obtained from cytological investigation of rosettes collected by centrifugation), 26% EAC rosettes with 20% cells carrying SIgM, 1 SIgG 2% SIgA, 3% SIgD, and no SIgE.

Polychemotherapy was initiated in March 1977 including daily doses of 2 mg vincristine, 600 mg cyclophosphamide and 100 mg fluorocortolone

cy of antigens HLA B7 and BW21 is suggestive for this unusual disease.

It is necessary to study new series of cases with PNH in order to make sure whether these antigens or other ones are found with a higher frequency in a statistically significant form.

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low to exclude that some of the initially found 'non-B/non-T' cells actually represented pre-B cells.

Unfortunately after this interesting phenomenon was observed, no additional, more sophisticated, investigations were possible since the patient died soon after. Our case shows, however, that more immunological and cytochemical follow-up studies should be done in Sézary syndrome before assuming that there exist different types of Sézary syndrome such as T cell or even B cell Sézary syndrome. We tend to believe that this is only one disease with different degrees of differentiation.

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Fig. 1. Two Sézary blood cells revealing their typical cerebriform nuclei. In one nucleus (right bottom), a nucleolus is surrounded by an opaque rim of nucleolus-associated chromatin.  $\times 10,500$

(modif 1) complemented with antibiotics and tuberculostatics. The further course was characterized by complete regression of skin lesions and recurrent partially haemorrhagic erythema, variable leucocyte counts between 5,000 and 56,000/mm<sup>3</sup> and up to 91% atypical lymphocytes. Treatment consisted of repeated course of chemotherapy with transient responses. The patient died 4 months after the diagnosis had been established with signs of pneumonia, cytomegalovirus infection, severe hyponatraemia and cardiac failure. Repeated cytochemical investigations in May 1977 revealed a few atypical tartrate-resistant acid-phosphatase-positive lymphocytes. Besides, a few Sézary cells had become PAS positive. Immunocytoologically 35% E rosettes were identified, all of which were atypical large lymphoblasts. B cells by rosetting tests and immunofluorescence were below 0.2% and SIg-negative.

## Discussion

The Sézary syndrome is generally thought to be a T cell malignancy [3, 4] al-

though there are a few reports on 'null-cell' characteristics of malignant cells in this disease [2, 7] as well as on T and B cell (clonemeral) characteristics [5]. Our case shows an apparent change in immunological characteristics from non B/non T cell to T cell malignancy in terms of E rosette formation with a slight increase in PAS stainability and occurrence of tartrate-resistant acid phosphatase in the atypical cells. This may indicate a certain degree of maturation of these atypical lymphoid cells during chemotherapy or a selective process in favour of more differentiated T cells. Since immature, fetal type T cells may also contain SIgM [being E rosette-negative 6] it cannot be excluded that some of the IgM carrying cells counted before chemotherapy were actually fetal-type T cells. This suggestion is based on the observation that atypical T cells later on increased during the course of the disease. The data as such, however do not al-

Acquisition of this book, as of its predecessors, is an absolute must for any research laboratory interested in up-to-date information on the biochemistry of blood coagulation, platelet physiology and pathogenesis of vascular disease.

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 Fischer Stuttgart 1979  
 XLIV + 503 pp., DM 48

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R. Burkhardt  
**Hämatologie**  
 Springer Berlin 1978  
 VIII + 140 pp. 8 fig. DM 4  
 ISBN 3-540-08901 2

Der Autor hat den Versuch unternommen, eine Hämatologie für den vielbeschäftigten praktischen

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C. R. Stiller J. B. Doxeyer N. R. Stclair and  
 F. T. Rapoport (eds)  
**Immunologic Monitoring of the Transplant Patient**  
 Grune & Stratton, New York 1978  
 XIX + 364 pp. US \$ 38.50  
 ISBN 0-8089-1124-4

This volume resulting from a symposium held in London, Oct. 1977 deals with all aspects of immunologic monitoring of the transplant patient.

The term "immunologic monitoring" covers a number of concepts, all concerned with changes

## Book Reviews

*A. E. Mourant A. C. Kopeč and  
K. Domaniewska-Sobczak*  
**Blood Groups and Diseases**  
*A Study of Associations of Diseases with Blood  
Groups and other Polymorphisms*  
Oxford Monographs on Medical Genetics  
Oxford University Press Oxford 1978  
328 p £25.00  
ISBN 0-19-264170-0

This book is a continuation of the second edition of *The Distribution of the Human Blood Groups* published by the same authors. The book has been divided into two sections. On the first 50 pages A. E. Mourant describes in brief chapters the association between particular blood groups (this term includes not only classical blood groups but all other genetic polymorphisms) and disease: neoplastic diseases, cancer of the lung, infective diseases, disorders related to pregnancy, peptic ulcers, diabetes mellitus, rheumatic fever, thrombosis, the phenylthiocarbamide tasting system, the alpha<sub>1</sub>-protease inhibitor system and the histocompatibility system. The main part (more than 200 pages) contains the tables of association between polymorphisms and disease including a report from the HLA and Disease Registry of Copenhagen, 1976. It is not possible to discuss in detail this vast body of data. The book provides valuable information for all those who are interested in the exciting field of blood groups and diseases. Dr Mourant and this team have to be congratulated.

S. Seidl, Frankfurt am Main

*Winslow S. Caughey*  
**Biochemical and clinical aspects of hemoglobin abnormalities**  
Acad. Press New York-San Francisco-London  
1978, XX + 725 p \$ 33.50  
ISBN 0-12-164350-6

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volume provides an excellent review of the knowledge on biochemistry, biophysics, pathophysiology and clinical implications of the most important hemoglobin disorders. It is a most valuable source of information for laboratory investigators and clinicians.

H. R. Marti, Aarau

*T. H. Spaet*  
**Progress in Hemostasis and Thrombosis, Vol. 4**  
Grune & Stratton, New York 1978  
XV + 410 pp., US\$ 34.50  
ISBN 0-8069-1096-5

This fourth volume of reviews concerning the pathophysiology of hemostasis and thrombosis contains a number of timely contributions on the interaction between the vessel wall, blood platelets, blood coagulation and the fibrinolytic system. The first chapter deals with transport mechanisms across the endothelial surface of arteries (Chien). It is followed by chapters on platelet contractile proteins (Adelstein and Pollard), properties of the platelet membrane (Shattil and Cooper), prostaglandins (Gerrard and White), initiation of the coagulation and fibrinolytic system (Kaplan) and an exhaustive review on prothrombin (Shapiro and Cord). Further contributions on measurement of fibrinogen derivatives (Wilner), interactions of platelets with injured vessel walls (Friedman and Burns), rheoptical studies of platelets (Frojmovic) and a critical assessment of the platelet survival time (Harker) provide a basis for indirect assessment of vascular disease. A final chapter on non-surgical management of acute nonvariceal upper gastrointestinal bleeding (Gilbert et al.) is somewhat outside of the general topic of this volume but of considerable clinical interest. The general presentation is outstanding, including many figures on the ultrastructure of the vessel wall and platelets. Quotations are numerous and include references up to 1978. Unfortunately references are not in alphabetical order, a minor drawback. If one considers that many chapters have more than 200 quotations, the plates on page II do not correspond to the description in the text and should be inverted.

Acquisition of this book, as of its predecessors, is an absolute must for any research laboratory interested in up-to-date information on the biochemistry of blood coagulation, platelet physiology and pathogenesis of vascular disease.

E. A. Beck, Bernae

G. Bondachuk und B. Schnorwies (Hrsg.)  
Immunologie. Ein Nachschlagewerk; 2. Aufl.  
Fischer Stuttgart 1979  
XLIV + 503 pp. DM 48.-

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### Causes of Death in Leukemia and Lymphoma with Modern Treatment

Bo Lantz, Jan Adolfsson, Bengt Lagerlöf and Peter Reizenstein

Division of Hematology, Departments of Medicine, Pathology and Oncology, Karolinska Hospital, Stockholm

**Key Words.** Cardiac death, Leukemia, Leukemic infiltration, Lymphoma, Septicemia

**Abstract.** An attempt was made to see if new forms of treatment of fungal and bacterial septicemia, or new cytostatic combinations have changed the causes of death in leukemia and lymphoma. Autopsies were studied of 16 cases with acute leukemia, 2 with chronic granulocytic leukemia and 24 with lymphoma. 10 of the 18 patients with leukemia and 10/24 lymphoma patients died although they had no, or only slight, tumor infiltration in the bone marrow and parenchymatous organs: only 1/18 leukemias had extensive infiltration. There was a statistically significant correlation between the extent of marrow and organ infiltration. Cardiac failure (5/18 patients) was almost as common as the main cause of death as septicemia (7/18) in leukemia. The corresponding figures in lymphoma were 5/24 and 9/24 respectively. The present findings contrast with earlier ones, where more septicemia and tumor infiltration were found at autopsy and less cardiac failure was observed. 15 of 16 cases with septicemia at autopsy had terminal fever. Bacteriological and histological signs of septicemia at autopsy agreed satisfactorily. There was a surprising absence of agreement between terminal granulocytopenia and septicemia: 13 of 16 patients with septicemia had over  $0.1 \times 10^9$  and 10 over  $0.5 \times 10^9$  granulocytes/liter blood.

Traditionally the main causes of death in leukemia are considered to be bacterial, mainly gram-negative septicemia and hemorrhage [5]. In addition, fungal septicemia is frequently found at autopsy [4]. However in some cases there is neither extensive leukemia nor septicemia, nor bleeding. In these

cases the cause of death is obscure. The present report has two purposes, to study the cause of death in these obscure cases, and to study the effect on fungal septicemia of the introduction of 5-fluorocytosine. For comparison a group of lymphoma patients is also investigated.

in graft recipient humoral or cellular immunity either donor-specific or nonspecifically functional, such as quantitation of T cell numbers or response to mitogen stimulation, etc. Other studies are directed at monitoring immunosuppressive therapy so as to optimize immunosuppression for individual patients. The book is divided into three sections. The first part contains excellent review papers dealing with the mechanisms of alloresponsiveness including contributions on the genetics of the major histocompatibility complex in man, the measurement of alloresponsiveness, the mechanisms of immunoregulation and the immune response to transplantation antigens. The second is devoted to the numerous methods for the measurement of pretransplant cell-mediated and humoral sensitization, with a few papers describing tests of immunologic competence. The

third section is concerned with the monitoring of posttransplant immune responses, and also includes several papers on the measurement of immunosuppression.

The book contains a considerable amount of interesting information for the immunologists involved in kidney or bone marrow transplantation. However it must be realized that many new immunologic tests developed in the hope of predicting the occurrence of various types of clinical graft rejection or assessing the degree of immunosuppression achieved, are still unreliable, and have led to contradictory conclusions. The multifaceted nature of immune responses can obviously not be monitored by one or two simple tests. Much remains to be done before immunologic monitoring becomes a safe guide for the management of the transplanted patient.

M. Jernst

## Bacteriology

Specimens for bacteriological cultures were taken from heart, blood, lung, liver and spleen. Fungal cultures were not performed.

## Cardiac Death

Heart and circulatory failure was diagnosed when indicated by the clinical picture and substantiated at autopsy by general congestion, pulmonary edema and dilatation of the left ventricle, with absence of other major causes of death.

## Results

### Leukemia

1 patient had no leukemic infiltration at autopsy and a normal bone marrow at microscopy 10 of the 18 patients had no or only slight leukemic infiltration in both marrow and other organs. This was consid-

ered insufficient to be the main cause of death. In 6 patients the leukemic infiltration was moderate and judged to be a contributory cause of death. In only 1 case was the leukemic infiltration extensive and judged to be the main cause of death.

Septicemia was the main cause of death in 7 of the 18 patients. The identified bacteria are shown in table II. 2 patients with *Escherichia coli* septicemia had intestinal leukemic infiltration. In 1 of these an intestinal erosion was seen in the infiltrated area.

2 patients had both septicemia and bleedings 1 from the urinary tract and the other from the stomach. Fungal septicemia was found in only 2 of the 18 patients, both



Fig. 1. Agreement between histological and bacteriological signs of septicemia in leukemia and lymphoma ( $n = 16$ ). 2 cases with deep mycoses excluded.

Table II. Bacterial septicemia at autopsy in 12/16 cases with leukemia and lymphoma

Organism	Number
Gram-positive bacteria	
<i>Staphylococcus aureus</i>	3
<i>Clostridium perfringens</i>	2
$\beta$ -Streptococcus	2
Gram-negative bacteria	
<i>Pseudomonas aeruginosa</i>	
<i>E. coli</i>	3
Bacteroides	1
<i>Salmonella</i> / <i>phlebotomus</i>	1

Combined septicemia.

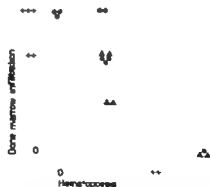


Fig. 2. Relationship between leukemic infiltration, hematopoiesis and cytostatic damage in triple bone marrow of 18 leukemias.  $\Delta$  = Patient with cytostatic marrow damage.  $\bullet$  = patient without marrow damage.

Table I. Description of patients

Diagnosis	Number of patients	Sex		Mean age years	Mean approximate duration of disease, years
		M	F		
<i>Leukemia</i> <sup>1</sup>					
Acute nonlymphocytic	14				
Acute lymphocytic	4	12	6	58	1 <sup>2</sup> (range 1 month to 4 years)
Chronic granulocytic	2				
<i>Lymphoma</i>					
Hodgkin	7				
Non-Hodgkin	14 <sup>3</sup>	12	12	59	5 (range 3 months to 17 years)
Chronic lymphocytic leukemia	3				

<sup>1</sup> Chronic lymphocytic leukemia is included in the lymphoma group in this study.

<sup>2</sup> Preleukemic state excluded in 2 patients.

<sup>3</sup> In 1 case acute lymphocytic leukemia developed.

## Material

All patients are described in table I. The study includes patients autopsied between September 1975 and February 1977 at the Karolinska Hospital.

14 consecutive patients with acute, nonlymphocytic leukemia, 2 with acute lymphocytic and 2 with chronic granulocytic leukemia were studied. 1 of the latter had a blastic transformation. 21 consecutive patients with lymphoma and 3 patients with chronic lymphocytic leukemia were investigated. 7 of them had Hodgkin's lymphoma and 14 non-Hodgkin's lymphoma. 2 patients with lymphoma did not receive cytostatics.

## Methods

Autopsy was performed according to a special schedule. Specimens for microscopy were taken from different organs including bone marrow, spleen, lymph nodes, liver, kidney, endocrine and neuroendocrine organs, heart, rectus femoris muscle, intestinal tract and brain.

The histological specimens were prepared for histology according to routine procedures. Sections were stained with hematoxylin-eosin and Giemsa. The sections were finally studied by two pathologists.

## Definitions

The histological definitions of fungal and bacterial septicemia in our earlier study were used [4]. A careful search was made for the presence of bacteria or mycelia in all tissues. Single or few bacteria or mycelia were disregarded, but foci or larger clumps in organs other than lungs or intestine were considered as histological evidence of septicemia.

The degree of leukemic cell infiltration was graded from 0 (none) to ++++. The leukemic infiltration was studied in all organs (general infiltration) and in the bone marrow (marrow infiltration) from 3 different locations: sternum, vertebra and femur. Aplasia was diagnosed if all three locations had fatty marrows, and no or very slight hematopoietic activity. The degree of hypoplasia and hyperplasia was recorded as well as evidence of cytostatic induced marrow damage. Cytostatic marrow damage was indicated by hemorrhages and edema in the marrow.

**Table IV** Cytostatic doses and bone marrow damage in 18 patients with acute and chronic granulocytic leukemia

Patient	Leukemic infiltration I triple bone marrow samples	Other findings	Approximate total cytostatic doses, mg
1	III	necrosis, signs of cytostatic damage	<i>Complete list</i> Rubicomycin 930, cytosine arabinoside 5,900, thioguanine 8,400, vincristine 21 methotrexate 300, permethol 400, cyclophosphamide 6,300
2	+	slight fibrosis, signs of cytostatic damage	rubicomycin 170, cytosine arabinoside 1,300, vincristine 7.5
3	++	signs of cytostatic damage	rubicomycin 350, cytosine arabinoside 2,230
4*	++	signs of cytostatic damage	rubicomycin 270, cytosine arabinoside 1,900, vincristine 2
5	+	severe cytostatic damage	rubicomycin 1,150, cytosine arabinoside 1,800, thioguanine 960
6	0	partly aplastic, signs of cytostatic damage	rubicomycin 270, cytosine arabinoside 1,900
All other patients ( = 12)		no visible damage	<i>Incomplete list (mean values)</i> rubicomycin 666 ( = 7), cytosine arabinoside 2,900 ( = 11), thioguanine 2,999 ( = 9), cyclophosphamide 4,800 ( = 6)

Concomitant myocardial necrosis [7].

**Table V** Fever prior to death in patients with or without septicemia at autopsy

	Fever °C	
	<38	38
Septicemia (n = 16)	1	15
No septicemia ( = 26)	13	12*

Including 4 cases with local infections (pneumonia, sinusitis, bronchitis), 4 cases with cerebral (7) fever (cerebral hemorrhage) and 4 with unknown cause of fever

Septicemia was the cause of death in 9 patients, 3 of whom also had severe hemorrhages. The identified microorganisms are shown in table II. All septicemias were bacterial. Only a local candida infection in the esophagus was found in 1 patient.

5 patients died from cardiac failure. 2 patients died from extensive tumor infiltration without accessory causes. Other causes of death were: respiratory insufficiency (3 cases), pulmonary abscess (1 case), increased intracranial pressure (1 case), ulcerative peritonitis (1 case), and meningitis (1 case). In 1 case the cause of death could not be determined.

Table III. Causes of death in acute leukemia

Study	Number of patients with acute leukemia	Septicemia <sup>1</sup> bacterial % <sup>2</sup> and fungal, %	Cardiac failure, %	No or slight general leukemic infiltration, %
<i>Herzk et al</i> [5]	366	71	8-23	4
<i>Chl et al</i> [4]	33	70	30	15
Present study (1975-1977)	16	38	13	31
				62

<sup>1</sup> Bleedings included.<sup>2</sup> Combined fungal and bacterial septicemia included.

with acute leukemia. Bacteriological and histological signs of septicemia agreed satisfactorily (fig. 1)

Cardiac failure was the main cause of death in 5 patients with acute leukemia who had only slight general leukemic infiltration. In addition cardiac failure was considered contributory in 1 patient with pneumonia. Hemorrhages occurred as the main cause of death in 4 patients. 3 were cerebral and 1 intestinal. Of the 3 patients with fatal cere

bral bleedings, 2 had leukemic infiltration in the brain

The findings in the marrow samples are shown in table IV and figures 2 and 3. As expected there was an inverse relationship between the presence of normal hematopoiesis and extensive leukemic infiltration (fig. 2). The bone marrow infiltration was significantly ( $p < 0.05$ ) correlated to the general leukemic infiltration of other organs (fig. 3). Bone marrow damage considered to be caused by cytotoxic drugs was found in 6 patients (table IV). 3 of these patients had basophilic necroses in the myocardium described elsewhere [7]. The mean cumulative rubidomycin dose was 523 mg in the patients with bone marrow damage, while 7 patients without signs of marrow damage had received 666 mg (table IV).

### Lymphoma

10 of the patients with lymphoma had no or slight general lymphoma infiltration which was considered insufficient to be a major cause of death. 6 patients had extensive tumor infiltration, and the rest had moderate infiltration.

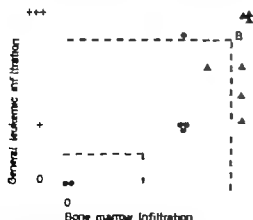


Fig. 3. Correlation between leukemic infiltration of triple bone marrow and that of other organs in 18 patients with leukemia. A is the mean of ● values and B that of ▲ values.  $A < B$  ( $p < 0.05$ )

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bral bleedings, 2 had leukemic infiltration in the brain.

The findings in the marrow samples are shown in table IV and figures 2 and 3. As expected there was an inverse relationship between the presence of normal hematopoiesis and extensive leukemic infiltration (fig. 2). The bone marrow infiltration was significantly ( $p < 0.05$ ) correlated to the general leukemic infiltration of other organs (fig. 3). Bone marrow damage considered to be caused by cytotoxic drugs was found in 6 patients (table IV). 3 of these patients had basophilic necroses in the myocardium described elsewhere [7]. The mean cumulative rubidomycin dose was 523 mg in the patients with bone marrow damage, while 7 patients without signs of marrow damage had received 666 mg (table IV).

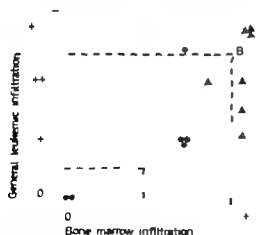


Fig. 3 Correlation between leukemic infiltration of triple bone marrows and that of other organs in 16 patients with leukemia. A is the mean of ● values and B that of ▲ values.  $A < B$  ( $p < 0.05$ ).

#### Lymphoma

10 of the patients with lymphoma had no or slight general lymphoma infiltration, which was considered insufficient to be a major cause of death. 6 patients had extensive tumor infiltration, and the rest had moderate infiltration.

myocardial cells has been described in the present patients [7]. 5 cases showed basophilic necroses in the myocardium, which is a reported sign of rubidomycin cardiotoxicity [2]. There was little difference in the total rubidomycin dose between the patients with extensive and those with little marrow damage. The finding of an individual susceptibility for rubidomycin marrow toxicity emphasizes the importance of individualization of the therapy.

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Table VI. Circulating granulocytes prior to death in 16 septicemic patients with leukemia and lymphoma

Granulocytes $\times 10^9/l$	Leukemia	Lymphoma
0 - 0.100	2	1
0.101-0.500	1	2
0.501-1.000	0	2
1.001-1.500	0	0
Over 1.500	4	4

### Relations between Clinical and Autopsy Findings

**Septicemia** There is good agreement between terminal fever and septicemia in leukemia and lymphoma (table V). 15 of 16 patients with septicemia had over 38 °C before death and 13/16 had over 39 °C. The patient without fever despite septicemia had the highest granulocyte count ( $27 \times 10^9/l$ ).

There is a surprising absence of agreement between granulocytopenia and septicemia if lymphoma and leukemia patients are considered jointly (table VI). No less than 13 patients with septicemia had more than 0.1 and 10 more than  $0.5 \times 10^9$  circulating granulocytes/liter blood [1].

## Discussion

### Tumor Infiltration

There was a significant correlation between general leukemic infiltration and bone marrow infiltration. However infiltration in the nervous system does not seem to be correlated to the general infiltration [12].

The present results show that about half of the patients with acute leukemia and lymphoma, given modern chemotherapy die without or with very limited general tumor infiltration. In acute leukemia the proportion of patients dying without or with slight

leukemic infiltration has increased significantly (table III) compared to our earlier study [4]. So have the frequencies of complete remission and the median survival time [6-9]. Taken together these findings suggest improvements in the treatment of the tumor disease itself; therefore appreciable effort should be devoted to the complications.

### Bacterial Septicemia

In the present study the frequency of septicemia as a cause of death in adult acute leukemia is less than that in the earlier study (table III). The difference is not statistically significant, perhaps because the number of patients in the study is small. Endogenous septicemia can occur when there is an intestinal erosion. This finding supports the use of intestinal sterilization [3, 6, 10, 11].

### Fungal Septicemia

The frequency of fungal septicemia has also decreased although not significantly. The decrease is probably due to the introduction in 1975 and since the last study [4] was concluded of 5-fluorocytosine to treat patients with symptoms of fungal septicemia [8].

### Cardiac Failure

Cardiac failure is not a commonly described cause of death in leukemia, although it is as common in the present material as septicemia. Cardiac failure may have become more important in this respect because the treatment of the leukemia itself and of other complications has been improved, and because rubidomycin is cardiotoxic. In addition to basophilic necroses, an increase in lipofuscin pigmentation of the

Table I. Details of cyclic oscillations

	Case number															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Duration of cyclic oscillation months	36	22*	9	3	4	2½	5½	7	2	2½	5	3	13	3½		
Number of cycles	22	10	7	3	3	1	3	5	1	2	2	1	8	2		
Type of cycles	S/R	S	S	R	R	S	R	S	R	R	R	R	R	R		
Period, days	20 100	50 93	15 90	15 35	25 35	67	50 70	15 80	60	77	45 105	90	30 60	40 65		
Amplitude, 10 <sup>3</sup>	10 100	17 225	5 20	10 65	15 40	35	20 50	15 11	40	15	19 36	52	15 45	24 58		
Follow-up	+	+	L	C	†	†	†	L	†	†	†	†	†	†	C	

= On hydroxyurea L = low to follow-up S = stationary = splenectomy C = blastic crisis R = rising † = persistently raised count requiring therapy + = continued oscillations.

## Discussion

Cyclic oscillations in the leukocyte count occur in healthy individuals, these cycles have a period of 14–24 days and an amplitude of 1,000–1,500 [4]. Cyclic oscillations of the leukocyte count have been reported in 12 cases of CML by different workers [2, 3, 5]. These oscillations cause difficulties in adjustment of the dose of drugs [3]. In 2 of our cases on hydroxyurea therapy the stationary-type cycles have continued for as long as 18 and 21 months. An attempt to withhold hydroxyurea led to rising cycles and reintroduction of the drug reverted the cycles to the stationary type. A similar phenomenon has not been observed with busulfan therapy. Cyclic oscillations of the leukocyte count being a physiological phenomenon, its preservation, even in a deranged form, may be considered advantageous. In

that case hydroxyurea may be a better form of therapy than busulfan for CML. This aspect needs to be studied by a controlled trial.

In cases of CML it is common practice to start treatment if the leukocyte count is over 30,000–50,000/mm<sup>3</sup>. The fall in the leukocyte count that occurs on starting treatment is attributed to the drugs. However the observations of spontaneous cyclic oscillations reported here as well as by other workers would imply that in a case of CML it is advisable to demonstrate a rising leukocyte count over a sufficient period of time before initiating treatment or altering the dose of the drug.

## Acknowledgement

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## Cyclic Oscillations in Leukocyte Count in Chronic Myeloid Leukemia

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**Key Words.** Busulfan Chronic myeloid leukemia Cyclic oscillations in leukocyte count Hydroxyurea

**Abstract.** Cyclic oscillations in the leukocyte count were observed on 14 occasions in 9 out of 70 cases of chronic myeloid leukemia. Importance of this knowledge lies in minimizing the total amount of drug therapy and avoiding frequent unnecessary alterations in the dose schedule.

In chronic myeloid leukemia (CML) the leukocyte count is an important parameter for monitoring the dose of drugs. Therefore, it would be of interest to be aware of spontaneous cyclic oscillations in the leukocyte count in CML.

### Material and Methods

Investigations were done using the standard methods [1]. Diagnosis of CML was based on high total leukocyte count with premature cells along with hepatosplenomegaly. When cyclic oscillations in leukocytes are seen, the time interval between two successive peaks or troughs expressed in days is called the period of cycle. The difference in the leukocyte counts at the peak and trough of the cycle is called the amplitude of the cycle. When the troughs of each successive cycle show normal or

near normal leukocyte counts, this is called a stationary type of cyclic oscillation whereas if the trough of each successive cycle shows a rising leukocyte count, this is called a rising type of cyclic oscillation.

### Results

Out of 70 cases of CML, 9 showed cyclic oscillations of the leukocyte count on 14 occasions. The details of these oscillations are shown in table I. 2 patients had more than one (3 and 4) episode of cyclic oscillations with a period of persistently raised leukocyte count requiring therapeutic intervention in between these episodes. In none of the patients were the peaks of the cycles associated with symptoms.

## <sup>99m</sup>Tc Labelled Human Leukocytes

### An *in vitro* Functional Study

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**Key Words.** Chemotaxis. Functional and metabolic tests. Human leukocytes. <sup>99m</sup>Tc

**Abstract.** A new method of human leukocyte labelling is described. 20 ml of human blood are allowed to sediment in a dextran solution and the remaining red blood cells are removed using NH<sub>4</sub>Cl. The leukocyte-rich suspension is incubated with a Sn-pyrophosphate solution, and then labelled, using 20 mCi <sup>99m</sup>Tc pertechnetate. Labelling efficiency is about 30%. Functional activity was shown to be satisfactory: (1) appearance under electron microscopy was quite normal; (2) *Klebsiella* ingestion, zymosan-stimulated oxygen consumption and iodination were similar in labelled and unlabelled cells, and (3) chemotaxis revealed no alteration in cell migration.

### Introduction

Several authors have used labelled phagocytes to detect inflammatory foci in cases of infection [9].

Cr was the first marker used but its high photon energy and long physical half-life are obvious drawbacks [7, 21]. Other gamma emitters such as <sup>67</sup>Ga have also been suggested for human leukocyte labelling [28] and for direct intravenous administration in abscess detection [6].

The criteria of a good labelling technique are the following: (a) high labelling efficiency; (b) high stability (activity should remain within the labelled cells); (c) high cell specificity (only polymorphonuclear phagocytes

should be labelled) and (d) normal viability especially as regards cell chemotaxis. In other words, labelled cells should be able to move to infectious foci as easily as unlabelled cells.

Recently, 3 labelling techniques for phagocytes were reviewed by McAfee and Thaler [19, '80]. The best results, at least in terms of efficiency, were obtained with <sup>99m</sup>Tc or In toxin complexes. However, these studies did not assay labelled cell viability [4, 6]. Reproducibility was therefore often poor and scintigraphic interpretation was sometimes difficult.

We have developed a new leukocyte labelling technique, used earlier for red cell labelling. In this method, cold tin pyro-

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should be labelled), and (d) normal viability especially as regards cell chemotaxis. In other words, labelled cells should be able to move to infectious foci as easily as unlabelled cells.

Recently 23 labelling techniques for phagocytes were reviewed by McAfee and Thakur [19-20]. The best results, at least in terms of efficiency were obtained with <sup>99m</sup>Tc or In 'oxin' complexes. However these studies did not assay labelled cell viability [4-26]. Reproducibility was therefore often poor and scintigraphic interpretation was sometimes difficult.

We have developed a new leukocyte labelling technique used earlier for red cell labelling. In this method, cold (in pyro-



phosphate is used as a first step since  $^{99m}\text{Tc}$  in pertechnetate form does not label any type of blood cells [19]. The aim of this leukocyte labelling technique was to obtain viable labelled phagocytes. So we have assayed a lot of quality control tests. More over the advantages of this technique compared to the others are discussed.

## Materials and Methods

### Preparation of Labelled Leukocytes

A leukocyte-rich suspension is required for labelling. 20 ml human venous blood was collected in a 60 ml sterile heparinized syringe (7% heparin-Choxy) containing 25 ml dextran (high molecular weight 500 000, Pharmacia, Uppsala, Sweden). The blood was allowed to sediment for 70–90 min at 20 °C with the syringe in a vertical position. The leukocyte-rich supernatant (40 ml) was then transferred to four conical plastic tubes. The remaining red cells were removed from this supernatant by adding 80 ml  $\text{NH}_4\text{Cl}$  0.87% solution. This solution was shaken vigorously and centrifuged for 5 min at 800 g. The supernatant was removed and the sediment washed using 9‰ NaCl and then centrifuged. The supernatant was again removed and the cells were resuspended in 4 ml NaCl. A leukocyte-rich suspension was thus obtained. The extraction efficiency index was obtained by a cell count and by calculating the ratio of leukocytes in this suspension to the initial number of leukocytes in the venous blood sample.

The suspension was then incubated for 15 min at 37 °C with a stannous pyrophosphate solution (59.4 mg pyrophosphate and 1.3 mg stannous chloride in a 3-ml solution), using 150 1/20,000 leukocytes. The free pyrophosphate was then removed by further centrifugation and washing. The resulting sediment was ready for labelling by incubation for 10 min at 20 °C together with 1 ml high specific activity pertechnetate solution (20–30 mCi/ml). The suspension was again centrifuged for removal of supernatant and free pertechnetate. After resuspension in NaCl solution, the sediment was ready for reinjection. The entire labelling procedure is sterile.

### Quality Control Methods

Quality controls consist of verifying the stability of the label and functional value of labelled cells. Stability is tested by the usual methods. We have measured the free  $^{99m}\text{Tc}$  in the suspension as follows: after centrifugation the free activity is counted three times, 5 min, 3 and 24 h after labelling. After the same intervals electrophoresis (in veronal buffer pH = 6.8,  $E = 100 \text{ V/cm}$ ) for 75 min, and paper chromatography (Whatman No 1 paper in methanol 85% water solution) were performed.

The value of the leukocytes was estimated by morphological study, chemotaxis and metabolic tests: cell morphology was examined by electron microscopy after cells have been fixed with 2.5% glutaraldehyde and included in epoxy resin (EPON). These microscope studies were made before and after incubation of the cells with stannous pyrophosphate, and after  $^{99m}\text{Tc}$  labelling. They were also performed after hypotonic lysis.

Chemotaxis was measured by the Cutler and Nelson technique [5–10]. Leukocyte migration was observed using a light microscope, after 2.5 h incubation at 37 °C. This test was also performed after each labelling step simultaneously with the stability test described above.

There are many metabolic tests for the study of the biochemical phenomena associated with phagocytosis and the death of pathogenic agents caused by polymorphonuclear cells. Of these, the following were applied: (1) spontaneous or stimulated oxygen production (by Zymosan) [17], (2)  $(\text{O}_2^-)$  superoxide anion production, measured by nitroblue tetrazolium reduction [2], (3)  $(\text{H}_2\text{O}_2)$  hydrogen peroxide production [23], and (4) iodination by  $^{125}\text{I}$  [15].

## Results

In general 40–45% of the leukocytes in the initial blood sample were recovered in the final suspension corresponding to  $50 \cdot 10^6$  leukocytes but only  $5 \cdot 10^6$  red cells. Relative to the criteria we defined above, the results are the following: Labelling efficiency was therefore 30–35% in normal blood. Under these conditions, the final sus-

Table I. Labelling stability *in vivo* (3 experiments) Chemotaxis and elution were simultaneously performed 5 min, 3 and 24 h after labelling. At 24 h, elution was about 25% and chemotaxis was suppressed.

Time after labelling, h	Elution, %	Chemotaxis of labelled cells	Chemotaxis of unlabelled control cells
0	1	+++	+++
3	20	+++	+++
24	25	0	0

Table II. Functional tests of the WBC

	Ingestion, number of <i>Klebsiella</i> associated with the granulocytes after 10 min	O <sub>2</sub> consumption, nanomoles of O <sub>2</sub> consumed per 10 <sup>6</sup> granulocytes/min		H <sub>2</sub> O <sub>2</sub> nanomoles of H <sub>2</sub> O <sub>2</sub> produced per 10 <sup>6</sup> granulocytes/ml	Iodination, nanomoles of iodide per 10 <sup>6</sup> granulocytes/h
		spontaneous	stimulated		
Labelled cells	4.8	3.3	11.8	5	8.6
	4.8	1.9	11.7	3.9	7.7
	4.3	1.5	8.7	3.1	12.3
Normal control cells	7.6	2	16	6.2	16
	± 2.5	± 1	± 2.5	± 2	± 4

limit 11

Three assays were performed with labelled cells. Results which are considered abnormal are those which fall at least within 5% significance limit.

pension of 3–10 ml exhibited an activity of about 8 mCi. **Labelling stability** Results are shown in table I. The label released at 24 h was about 25 %. The preparation should be used immediately after labelling. Paper chromatography eluted 2% free pertechnetate. After 4 h free pertechnetate migration was observed by electrophoresis, with a peak of approximately 70% (fig. 1). **Cell specificity** The high affinity of pyrophosphate for the red cells constitutes a serious problem. It is therefore imperative in this technique to remove the red cells before la-

belling. **Cell Function** The same chemotaxis was observed before and after labelling (fig. 3).

Electron microscopy revealed no major abnormality in the nuclei or the cytoplasm of most granulocytes. The only abnormality observed was a relative degranulation of neutrophils, although eosinophils were normal.

The metabolic test values were at the lower limit of normal, and the viability of these labelled white cells was thus obvious (table II).

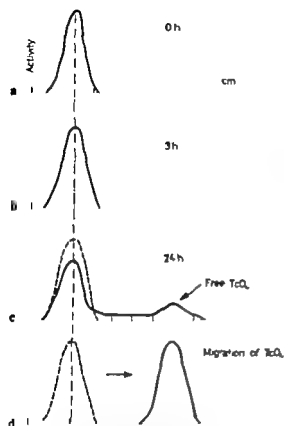


Fig. 1 Electrophoresis of  $^{99m}\text{Tc}$  labelled leukocytes. Almost all radioactivity was recovered just after labelling (a) or 3 h later (b) in the area in which labelled cells were deposited. 24 h after

## Discussion

Since each step in the leukocyte labelling technique involves some degree of functional damage, it cannot be discussed without reference to cell viability and specificity

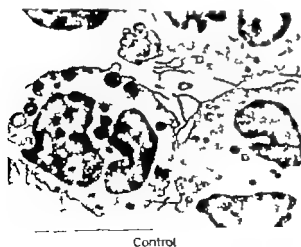
### Cell Specificity

The separation technique used resulted in a final product rich in granulocytes but the monocytes were not eliminated. However since the number of polynuclear cells as high compared to the other white blood cells in infected patients, this technique can easily be used

The important role of the red cells is shown by comparing two suspensions (table III)

The first suspension was obtained from a venous blood sample in dextran and contained both red and white cells (see labelling procedure), be

labelling about 20% displacement of free pertechnetate was observed (c) Electrophoresis showed most of the activity in strip lengths of 5–8 cm (d).



Control



After labeling

Fig. 2. Morphological appearance of PMN before and after labelling. Electron microscopic

study showed normal appearance with relative cytoplasmic degranulation of labelled cells.

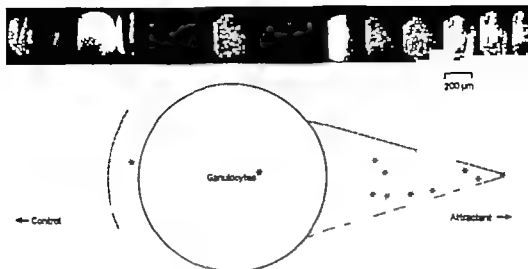


Fig. 3. Direct observation of chemotaxis. The attractant used is the supernatant of an avirulent *Klebsiella* culture. After 24 h incubation at 37 °C, PMN migration is compared to spontaneous control incubation. This photograph shows

PMN migration towards the attractant as observed by light microscopy. On the left very few cells are visible near the central hole, in which PMN were initially deposited.

Table III. Relative importance of the labelled red cells

	Leukocyte-enriched suspension	Red cell suspension	WBC
Leukocytes	20·10 <sup>6</sup> /l 5·10 <sup>9</sup> total	0	20·10 <sup>6</sup> l
Red cells	140·10 <sup>6</sup> l 5·10 <sup>9</sup> total	150·10 <sup>6</sup> l	0
T-Labeling efficiency	67 ± 6% (6 experiments)	45 ± 3% (3 experiments)	37 ± 5 (10 experiments)

Comparison of the labelling efficiency in three suspensions prepared using the same technique. More than half the total activity was taken up by the red cells.

fore red cell lysis. The second suspension, as obtained directly from globular pellet and contained red cells only their number being the same as in the first solution. Labelling efficiency was 67% in the first solution and 45% in the second. This shows that the label was not selective.

Several methods of removing red cells without damaging white cells have been described in the literature. Of these, mechanical removal of the red ring on the top of the sediment did not prove satisfactory [4],

## Discussion

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### Cell Specificity

The separation technique used resulted in a final product rich in granulocytes but the monocytes were not eliminated. However since the number of polynuclear cells is high compared to the other white blood cells in infected patients, this technique can easily be used.

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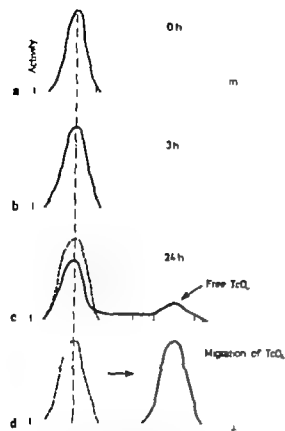


Fig. 1 Electrophoresis of  $^{99m}\text{Tc}$  labelled leukocytes. Almost all radioactivity was recovered just after labelling (a) or 3 h later (b) in the area in which labelled cells were deposited. 4 h after



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Table IV Different hypotonic and immunological lyses were performed and chemotaxis was assayed simultaneously (four experiments)

Method for RBC removal	Mean WBC, number/l	Chemotaxis (0 to +++)
1 Immunological (anti-A serum)	$5,600 \cdot 10^6$	+
2 Hypotonic dilution ( $H_2O$ ) (in dextran suspension)	$15,000 \cdot 10^6$	+
3 Hypotonic dilution ( $H_2O$ ) (after centrifugation)	$30,000 \cdot 10^6$	0
4 $NH_4Cl$ 0.87% solution	$27,500 \cdot 10^6$	+++
5 No RBC removal	$30,000 \cdot 10^6$	+++

The best result, i.e., RBC disappearance, preservation of all WBC and normal chemotaxis, was obtained in experiment 4.

Table V Efficiency of leukocytes labelling and chemotaxis assays using different amounts of  $3\alpha$  pyrophosphate solution

Pyrophosphate SN amount per 20,000/cells	Labelling efficiency %	Chemotaxis
50 $\mu$ l 2 assays	$17 \pm 2$	+
100 $\mu$ l 2 assays	$19 \pm 2$	+
150 $\mu$ l 2 assays	$29 \pm 4$	+
200 $\mu$ l 2 assays	$33 \pm 1.8$	-

Efficiency tests were carried out using the same leukocyte preparation. Two determinations were made for each amount of pyrophosphate tested (3 tubes per test). The SD was calculated from 4 experiments.

centrations of up to 200  $\mu$ l/20,000 cell-labelling efficiency can be slightly raised but the enzymatic properties and mobility of the cells labelled decrease significantly.

**Homogeneity** Dilutions of up to 1/200,000 showed no heterogeneity in radioactivity distribution but more sophisticated studies, especially autoradiography are nevertheless necessary to verify labelling homogeneity and for more accurate definition of labelling sites.

**Stability** There is an apparent discrepancy between the results for 24 h elution and those obtained by chromatography and electrophoresis. Two explanations can be suggested, firstly dilution of the cell suspension in a NaCl solution may further damage

cells which have already been diluted and centrifuged several times, and secondly the discrepancy might be the result of cell death which at ambient temperature is higher than 50% in this connection, it is difficult to distinguish between elution and cell death [1].

It should be stressed that labelling stability *in vitro* can be quite different from that observed *in vivo*. Studies using a different labelling method [3] showed a large uptake of labelled <sup>99m</sup>Tc cells in bone, and in the reticuloendothelial system. This might either be due to agglutination [4] or to macrophagic captivation of damaged white cells [16]. Better results should be obtainable if the viability of the reinjected cells is improved. In any case, it seems important to

and gramicidine lysolecithin purification resulted in a considerable loss of polymorphonuclear cells [21]. Several chemical or immunological methods were also tried and the results are shown in table IV. Some of these methods greatly reduced the number of white cells, others impaired chemotaxis. The most satisfactory was the  $\text{NH}_4\text{Cl}$  method as described by *Gray et al* [14] which was the technique chosen for this study.

Platelets were of course present in our final suspension (a very small number was observed on the microscope photographs) but it was not possible to ascertain their fate during separation. Although platelets can be eliminated by centrifugation at 1,200 g we were unable to establish that the advantage of such separation outweighed the risks of cell deterioration it might involve.

### Viability Tests

Most authors only use trypan blue tests to assess the viability of labelled white cells. Unfortunately this method is very inadequate for viability estimation since only the dead cells are colored. However among the living cells which are not colored, there is no distinction between normal cells and those which are on the verge of dying and therefore devoid of any functional capacity. This explains the need for the assessment of *in vitro* cell viability such as the electron microscopic studies, chemical tests of the enzyme activity and chemotaxis described here. The results obtained for labelled cells are satisfactory but not completely normal. Electron microscopic studies showed relative cytoplasmic degranulation. Cell membrane studies are now in progress, since phagocytosis seems connected with cytoplasmic and membrane granulations [16]. Values for enzymatic activity tests are at the

lower limit of normal and correlate well with the degranulation observed. Ingestion deficiency is associated with a deterioration of all subsequent oxygen-dependent metabolic processes (table II). This proves that the separation and labelling procedures as a whole may damage the cells, despite the precautions taken.

Chemotaxis of the labelled cells was positive, but raises an important question. Are the cells which are observed to move, labelled or not? Another possibility is that they might not be labelled homogeneously in which case unlabelled cells still move towards the attractant, but labelled cells could not.

Very small samples of the migration support (gelosils) were selected at several points. Mean counts for samples on the Klebsiella slide were 5–10 times higher than on the other side. This proves that the moving cells are definitely labelled, but we are now trying to obtain more accurate measurements.

Bacteriophage tests were not used, since this only appears after migration.

### Labelling Performances

**Efficiency** The purpose of the labelling technique is not simply to obtain the highest possible activity. Although this activity must be high enough to be easily counted and provide scintigraphic images, it is far more important for the method to leave the cells intact. The use of high specific activity is important. Labelling efficiency can vary from 10% with a 2 mCi/ml solution, to 35% using a 20-mCi/ml solution. Higher labelling efficiency values may be obtained but unhappily result in decreased functional activity of labelled cells (table V).

In fact, for higher pyrophosphate con-

Table IV Different hypotonic and immunological lyses were performed and chemotaxis was assayed simultaneously (four experiments)

Method for RBC removal	Mean WBC, number/l	Chemotaxis (0 to +++)
1 Immunological (anti-A serum)	5,600-10 <sup>6</sup>	+
2 Hypotonic dilution (H <sub>2</sub> O) (in dextran suspension)	15,000-10 <sup>6</sup>	+
3 Hypotonic dilution (H <sub>2</sub> O) (after centrifugation)	30,000-10 <sup>6</sup>	0
4 NH <sub>4</sub> Cl 0.87% solution	27,300-10 <sup>6</sup>	+++
5 No RBC removal	30,000-10 <sup>6</sup>	+++

The best result, i.e., RBC disappearance, preservation of all WBC and normal chemotaxis, was obtained in experiment 4.

Table V Efficiency of leukocytes labelling and chemotaxis assays using different amounts of a 8α pyrophosphate solution

Pyrophosphate 8N amount per 20,000/cells	Labelling efficiency %	Chemotaxis
50 μl 2 assays	17 ± 2	+
100 μl 2 assays	19 ± 2	+
150 μl 2 assays	29 ± 4	+
200 μl 2 assays	33 ± 1.8	-

Efficiency tests were carried out using the same leukocyte preparation. Two determinations were made for each amount of pyrophosphate tested (8 tubes per test). The SD was calculated from 4 experiments.

centrations of up to 200 μl/20,000 cell-labelling efficiency can be slightly raised but the enzymatic properties and mobility of the cells labelled decrease significantly

**Homogeneity** Dilutions of up to 1/200,000 showed no heterogeneity in radioactivity distribution but more sophisticated studies, especially autoradiography are nevertheless necessary to verify labelling homogeneity and for more accurate definition of labelling sites.

**Stability** There is an apparent discrepancy between the results for 24 h elution and those obtained by chromatography and electrophoresis. Two explanations can be suggested firstly dilution of the cell suspension in a NaCl solution may further damage

cells which have already been diluted and centrifuged several times, and secondly the discrepancy might be the result of cell death which at ambient temperature is higher than 50% in this connection, it is difficult to distinguish between elution and cell death [1]

It should be stressed that labelling stability *in vitro* can be quite different from that observed *in vivo*. Studies using a different labelling method [3] showed a large uptake of labelled <sup>99m</sup>Tc cells in bone, and in the reticuloendothelial system. This might either be due to agglutination [4] or to macrophagic captivation of damaged white cells [16]. Better results should be obtainable if the viability of the injected cells is improved. In any case, it seems important to



use the labelled cells immediately after their preparation. If they are re injected into the patient, pictures should be made during the 12 h that follow.

#### *In vivo Utilization*

Polymorphonuclear cells viability cannot be assessed completely by only using *in vitro* tests. *In vivo* studies remain of course necessary to fully appreciate the functional value of these cells. Moreover results may be different from normal and for blood from infected patients or patients with hematological diseases. Labelling efficiency greatly depends on the number of circulating leukocytes. We have not yet measured this number accurately in man but, in dogs, we found that with the same labelling method, efficiency was  $48 \pm 4\%$  for 40 000 leukocytes/ $\mu$ l. In dogs with turpentine-induced abscesses, labelling efficiency rose to  $70 \pm 5\%$  for 70 000 leukocytes/ $\mu$ l.

In addition, the functional properties of white cells may vary from one patient to another and are probably very different in normal and infected patients. This is strongly suggested by several studies of the inflammatory process, especially of the chemotaxis-inhibiting factor [12, 27] but this factor was not studied here. However there is reason to hope that the use of labelled white cells might provide a better quantitative means for studying inflammatory phenomena.

#### *Comparisons to Other Labelling Techniques*

The Oxin-In method, as described by Thakur *et al* [25] is now widely used [8, 13, 24]. Uptake of the labelling agent is high and it remains strongly bound to the cells [19, 20]. The chemical pattern of the uptake is well

understood [25]. However this technique means placing the living cells for quite a long period in an acid medium, corresponding to very unphysiological conditions. Extraction of the Oxin-In complex is a long process. In addition, the radiotoxicity of the In label caused by Auger electrons seems fairly high [18]. The dose reaching the spleen after *in vivo* administration has been estimated to be 18–20 rad/mCi [11]. Cell damage to lymphocytes and platelets has been studied, but not in polymorphonuclear cells [22].

The  $^{99m}\text{Tc}$  method chosen here is not completely atoxic [22] but toxicity to labelled cells seems low [11] and the functional behavior of the leukocytes does not seem affected. The Oxin  $^{99m}\text{Tc}$  method was not tested.

A satisfactory system of verification has not yet been evolved for any of the labelling methods used so far.

#### *Conclusion*

The use of a new separation and labelling technique with  $^{99m}\text{Tc}$ , enabled preparation of a leukocyte-rich suspension in which the polymorphonuclear cells were viable. This suspension can be used for two purposes: firstly to quantify inflammatory processes and other phenomena including cell migration, and secondly to detect infections or inflammatory foci using scintigraphic techniques. To this end, *in vivo* imaging procedures on animals are now being tested.

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## Defective Stimulating Capacity of Leukocytes in Mixed Leukocyte Culture in Constitutional Aplastic Anemia Caused by Suppressor T Cells

### A Case Study

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**Key Words.** Aplastic anemia Mixed leukocyte culture Suppressor T cells

**Abstract.** A patient with constitutional Fanconi-like aplastic anemia who developed a preleukemic state was transplanted with bone marrow from his HLA-identical MLC-negative brother. Before transplantation his mononuclear leukocytes were found to have a defective stimulating and responder capacity in mixed leukocyte culture with cells of an unrelated individual. After removal of T cells the stimulating capacity was completely restored. MLC testing with separated cell fractions seems important to establish HLA-D identity with a possible donor, especially when MLC tests with unseparated cells are negative.

### Introduction

Aplastic anemia is a disorder characterized by peripheral blood pancytopenia associated with a lack of bone marrow precursor cells. In severely affected patients there is a high mortality mainly caused by hemorrhage and infection. In the constitutional variant of aplastic anemia (e.g., Fanconi's anemia) development of leukemia is a substantial risk. In recent years transplantation of bone marrow from a healthy donor matched at the major histocompatibility complex (MHC) has become an accepted form of therapy in aplastic anemia [5-17] and to some extent also in leukemia [3-

18]. The first step of testing for MHC identity between patient and potential donor is done by determining the serologically defined HLA antigens coded for by the A, B and C loci of the MHC. When these are found to be identical D locus identity is established by performing a mixed leukocyte culture (MLC) test. In this test patient's mononuclear leukocytes (MNL) are cultured together with MNL of the HLA-identical donor. Differences in membrane antigens between patient's and donor's MNL, which are supposed to be important in determining the outcome of marrow transplantation [21] induce lymphocyte proliferation in MLC testing. Thus, a negative MLC, i.e.,

absence of proliferation, can be interpreted as indicating identity in these MNL membrane antigens. This interpretation is subject to the assumption that these antigens in the patients concerned have normal stimulating capacity.

Recently however we observed a patient with a preleukemic state following constitutional Fanconi like aplastic anemia, whose unseparated MNL failed to stimulate allogeneic cells in a MLC and also failed to respond on stimulation by these cells. After removal of T cells from the patient's MNL, the stimulatory capacity was completely restored. Because it seems possible to unmask false negative MLC studies in this situation by performing MLC testing with separated cell fractions, this case study is reported.

### Case Report

A 21-year-old white male was first admitted to the Pediatric Department of our hospital at the age of 12 because of anemia. Peripheral pancytopenia was found. At some occasions bone marrow examination showed hypocellularity and at others a varying cellularity. However a deficiency of megakaryocytes was found constantly. In addition, several congenital abnormalities were observed: thoracic scoliosis and cervical spine deviations, high arched palate, vascular abnormalities and hypogenitalism. In contrast with the classical Fanconi syndrome [2, 14], dwarfism, mental retardation with microcephaly, microphthalmus, kidney malformations, spleen atrophy, radius and thumb deformities and skin hyperpigmentation were not present. Family studies also were negative. Chromosomal instability considered to be a frequent symptom in Fanconi's anemia [14] was observed on chromosomal analysis in 1974. In 1977 this study was repeated and now an abnormal metacentric chromosome belonging in size to the III group was found on the location of the eleventh chromosome.

A diagnosis of 'constitutional Fanconi-like aplastic anemia' was made. Treatment with oxy-

metholone was started in 1969 with favorable response on the Hb level, and discontinued in March 1977 because of abnormal liver function tests. In August 1977 when these tests were already normal, androgen therapy was reinstated because of an increasing need for transfusion of red cells and platelets. Pancytopenia, however persisted. HLA typing of the patient and his family was performed to look for a potential donor for bone marrow transplantation. A 15-year-old male sibling was found to be HLA-identical with the patient. At the time of this study the following characteristics were found in patient's peripheral blood and bone marrow: platelet count 3,000/mm<sup>3</sup>, granulocyte count 450/mm<sup>3</sup>, MNL 750/mm<sup>3</sup>, Hb 6.3 g/dL. MNL consisted of 68% lymphocytes and 32% monocytes (by nonspecific esterase staining). Lymphocytes consisted of 75% thymus-derived (T) cells (as determined by spontaneous rosette formation with sheep red blood cells) and 16% bursa equivalent-derived (B) cells (detected by using a fluorescent polyvalent antihuman immunoglobulin serum). Immunoglobulin levels were within the normal limits. The bone marrow was hyperplastic by cytology and histology. There was a marked shift to the left, with a great excess of myeloblasts, erythroid hyperplasia with striking dyserythropoiesis and virtual absence of megakaryocytes.

*In vitro* cultures of the patient's and the donor's marrow were performed simultaneously (10<sup>4</sup> cells in methylcellulose as described by Iscove et al. [8] with  $\pm$  1 unit erythropoietin - human urinary-ep step III - and with 20% human leukocyte conditioned medium). Colonies formed by erythroid (CFU-E and BFU-E) and myeloid precursors (GM-CFC) were scored on day 7 for CFU-E and on day 14 for the other colonies. Patient's cultures showed a growth pattern as seen in acute myelogenous leukemia: no normal-sized colonies were observed, innumerable myeloid and erythroid clusters of 10-20 cells were seen in contrast to the donor's marrow which grew a normal number of normal-sized colonies. Bone marrow transplantation was considered to be indicated at that moment because of the severity of pancytopenia, requiring multiple transfusions of red cells and platelets, and the existence of a preleukemic state in a patient high at risk of developing acute leukemia. The patient was conditioned for transplantation with cyclophosphamide, 3.2 g daily

from day -5 to day -1. Transplantation was performed with  $7.2 \times 10^6$  bone marrow cells per kilogram. Engraftment was successful. However moderate graft-versus-host disease (GVHD) developed. The clinical course was complicated by recurrent bleeding from duodenal and gastric ulcers, for which surgical therapy was necessary. After this procedure the patient developed respiratory and cardiac failure and died 3 months after transplantation. Autopsy was not performed.

## Materials and Methods

Freshly drawn heparinized blood was obtained from the patient, HLA-identical brother and an unrelated individual.

### *Lymphocyte Isolation and Separation*

MNL cells were obtained by Ficoll-Isopaque gradient centrifugation [4] and are referred to as unseparated cells. T and non-T cell populations were obtained by separating E rosettes on Ficoll-Isopaque of higher specific gravity (1.090), as described by Parfitt [13]. Briefly unseparated cells were mixed with SRBC and layered at once on Ficoll-Isopaque (specific gravity 1.090) and centrifuged at 1,000-1,600 g with rapid acceleration at 20 °C. The rosetting cells which appeared in the red cell pellet were freed from erythrocytes by lysis with 0.91% ammonium chloride for 5 min at 11 °C. For effectiveness, the separation procedure was repeated for the non-T cells. The non-T cell fraction contained < 3% T cells.

### *Lymphocyte Cultures*

Cells were washed three times in RPMI 1640 with HEPES buffer (Gibco Biochemicals) supplemented with penicillin-streptomycin. Cultures were performed in round bottom microtitre plates (Corning, U-form, 220 M 24 AR) in final volume of 0.10 ml of the above-described medium with 25% inactivated human serum. Mitogenic stimulation was done on  $3 \times 10^4$  MNL per well with PHA (Wellcome, stock solution consisted of 1 ampoule PHA dissolved in 5 ml distilled water) in concentrations of 1 and 5  $\mu$ l/ml and with Con A (Calbio-

chem) in concentrations of 1, 3 and 10  $\mu$ g/ml. For antigenic stimulation 25  $\mu$ l of an antigen cocktail solution in medium (containing PPD in final concentration of 5  $\mu$ g/ml, diphtheria toxoid 10 IU/ml and tetanus toxoid 10 IU/ml) was added to  $10^5$  MNL per well.

For MLC testing  $1 \times 10^6$  responder cells were mixed with  $1 \times 10^6$  stimulating cells. T inhibit proliferation stimulating cells were treated before with mitomycin (Sigma Chemical Co.), 50  $\mu$ g/ml, during 30 min and afterwards washed three times in medium to prevent the carry-over of mitomycin.

All cultures were carried out in triplicate. Control cultures without stimulant were included. Plates were covered by a hard lid and wrapped in plastic adhesive kitchen film. They were incubated at 37 °C in humidified 5% CO<sub>2</sub> in air atmosphere for 5 days (PHA and Con A stimulation) or for 6 days (antigen stimulation and MLC test). 0.5  $\mu$ Ci of <sup>3</sup>H-thymidine (Radiochemical Centre, Amersham, England) was added 16 h before harvesting. Harvesting was performed with a multiple cell culture harvester (Skatron, Oslo, Norway) using glass fiber filters. The dried (60 min at 60 °C) filters were transferred to counting vials, 4 ml of scintillation fluid were added and counting was performed in liquid scintillation counter. The results are expressed as disintegrations per min (dpm) per well. The arithmetic mean and standard deviation of triplicate cultures were calculated.

## Results

The ability of the patient's and his brother's MNL to react to plant mitogens and to recall antigens was studied (table I). The reactivity of patient's MNL to recall antigens is strongly depressed. Concerning the reactivity to plant mitogens, there is a strong dissociation between the response to PHA, which is normal, and the response of patient's cells to Con A, which is strongly depressed. The MNL of the patient's brother had a normal proliferation capacity on plant mitogens and recall antigens. In the



mixed leukocyte culture (table II) the patient's MNL apparently do not give a proliferative response on stimulation with unrelated cells, whereas the MNL of the HLA-identical sibling show an excellent response when stimulated by the same unrelated cells. The patient's cells also do not stimulate unrelated cells. Again the stimulating effect of his brother's MNL on allogeneic cells is adequate. To test if the T cell frac-

tion had a suppressive effect on the stimulatory capacity of the patient's non T cells, a T/non T separation procedure was performed. Following this separation MLC testing between the patient's separated cell fractions and the unseparated cells of the HLA-identical sibling and the unrelated person was done (table II). The patient's non T cells were found to have a strong stimulating effect on unrelated cells, whereas no stimulation occurred in a MLC with cells of his HLA identical brother. After reconstitution of the patient's T and non-T cells the same results were obtained in MLC testing as with unseparated cells (table II).

Table I. MNL reactivity to plant mitogens and recall antigens

	Patient MNL (A)	MNL of HLA-identical sibling (B)
PHA, 5 $\mu$ l/ml	31,391 $\pm$ 382	27,829 $\pm$ 606
PHA, 1	4,975 $\pm$ 374	4,486 $\pm$ 651
Con A, 10 $\mu$ g/ml	1,090 $\pm$ 534	21,570 $\pm$ 747
Con A 3	1,195 $\pm$ 227	14,157 $\pm$ 496
Con A 1	902 $\pm$ 349	7,489 $\pm$ 1,320
blank	88 $\pm$ 13	129 $\pm$ 5
Antigen cocktail	452 $\pm$ 229	13,777 $\pm$ 1,357
Blank	278 $\pm$ 58	749 $\pm$ 35

Reactivity is expressed as mean dpm of triplicate cultures with standard deviation.

## Discussion

Bone marrow transplantation was performed in this patient with a preleukemic state in a constitutional dysplastic marrow. The outcome on any marrow transplantation is strongly influenced by differences in histocompatibility between donor and acceptor. So prior to transplantation, after matching for the serologically defined HLA antigens, MLC testing has to be done with

Table II. MLC tests in patient (A), his HLA-identical brother (B) and an unrelated individual (C) effect of removing and readding T cells on the stimulating capacity

Responder cells ( $1 \times 10^4$ )	Stimulating cells ( $1 \times 10^6$ )				B	C
	A	non-T cells A	T cells A ( $0.5 \times 10^6$ ) + non-T cells A ( $0.5 \times 10^6$ )			
A	61 $\pm$ 39	101 $\pm$ 37	173 $\pm$ 41		104 $\pm$ 5	158 $\pm$ 68
B	109 $\pm$ 24	575 $\pm$ 162	217 $\pm$ 53		1,340 $\pm$ 469	7,779 $\pm$ 2,650
C	1,390 $\pm$ 232	27,776 $\pm$ 6,249	1,690 $\pm$ 376		27,727 $\pm$ 1,775	1,817 $\pm$ 710

Stimulating cells were treated before with mitomycin 50  $\mu$ g/ml.

Results are expressed as mean dpm of triplicate cultures with standard deviation.

the MNL of the patient concerned and the possible donor in order to check for HLA-D identity. However Twomey *et al.* [20] reported that the MNL from 4 of 6 patients with aplastic anemia failed to stimulate the MNL from unrelated individuals in a macrophage-poor MLC, but did stimulate in a macrophage-enriched culture. In addition, Mickelson *et al.* [12] demonstrated a diminished or absent stimulating capacity on allogeneic cells in 28 of 53 patients with aplastic anemia, whereas the responder capacity was normal in these patients. Negative data in MLC tests are therefore not always sufficiently informative. Our patient's leukocytes also did not stimulate allogeneic cells. A relative absence of cells with stimulating capacity in MLC seems unlikely as the percentage of monocytes, B- and T-cells was normal in the peripheral blood of this patient. The results of cell separation studies demonstrated that the defective stimulating capacity of his cells on allogeneic cells was completely restored by removing T cells from his unseparated cells. The patient's non-T cell fraction provided effective stimulation of unrelated responder cells, whereas cells of his HLA-identical brother were not stimulated. So HLA D identity could be established by performing MLC testing with separated cell fractions. Our patient had both diminished stimulating and responder capacity in the MLC test. As far as the stimulating capacity is concerned it is obvious from reconstitution experiments that the T lymphocytes of our patient were capable of inhibiting the stimulating capacity of his own non-T cells. Whether we are dealing here with a T cell subpopulation with a specific suppressive effect for the mixed-leukocyte reaction is not clear. Three investigators recently documented the presence of a

suppressor T cell specific for the mixed leukocyte reaction in man [1 6 11]. A genetic restriction in this suppression, in which the suppressor T cell recognizes determinants on the stimulator as well as I locus products on the responder cell, was demonstrated in one study [6]. These suppressor cells were found in healthy humans without evidence of immunological or hematological abnormalities.

However our patient had a defective responder capacity towards allogeneic cells, to recall antigens and to the mitogen Con A, whereas the response on stimulation with PHA was normal. One might speculate whether suppressor cell activity was also responsible for hyporesponsiveness on stimulation with antigens and with Con A. As shown by Streikauskas *et al.* [16] T suppressor cells are activated by Con A, these cells were different from T cells, which proliferate on stimulation with Con A, PHA stimulated T cells which function as helper cells after stimulation. The discrepancy between the normal response to PHA and the absent response to Con A in our patient may therefore be explained by an increased number of suppressor cells in his peripheral blood MNL. The occurrence of suppressor cells in the peripheral blood with the capacity to inhibit erythro- and myelopoiesis of normal stem cells has been documented in some patients with aplastic anemia [7 9 10]. Recent reports suggest that the generation of suppressor cells in these patients was a consequence of previous blood transfusion [15 19]. Our patient received multiple transfusions. The question arises if the generation of suppressor cells for the MLC was also a consequence of these transfusions. We cannot exclude this possibility although we did not observe this phenomenon in oth-

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er multiple-transfused patients. At the moment it seems important to realize that suppressor T cells can give false-negative results in MLC testing. Our study indicates that in this situation MLC tests with separated cell fractions may be worthwhile.

## Acknowledgement

The chromosome analysis was performed by Prof. Dr G J P A Anders Department of Cytogenetics, State University Groningen (The Netherlands).

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## A Subpopulation of Lymphocytes with T and B Cell Characteristics

### Observations on Two Different Cases of Immunodeficiency

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**Key Words.** Lymphocyte subpopulations Mitogens Wiskott Aldrich syndrome  
Common variable immunodeficiency

**Abstract.** We present the immunological studies on 2 patients: one with the Wiskott Aldrich syndrome and another with common variable hypogammaglobulinemia. Although these patients represent two different immunodeficiency defects, both had a subpopulation of peripheral lymphocytes which had simultaneously T and B characteristics. Their peripheral blood mononuclear cells had a low responsiveness to polyclonal mitogens. We suggest that these unusual cells represent a subpopulation of suppressor cells or of immature T cells, which may have an essential role in the pathogenesis of these immunodeficiencies.

### Introduction

Primary immunodeficiency results from a failure to express an efficient humoral and/or cellular immune response. The Wiskott Aldrich syndrome (WAS), which is the least understood entity among the immunodeficiencies, is characterized by thrombocytopenia, eczema and very low serum IgM levels. Several elements of the effector limb of both humoral and cellular immunity seem to be intact. The inability to raise an immune response after immunisation implies a defect at the beginning of the immune response, i. e., at the antigen recognition and procession stages [3 4 6]. The common variable immunodeficiency (CVI)

is characterized by severe hypogammaglobulinemia involving all immunoglobulin classes. Clinically there are recurrent infections and lymphoid neoplasia or autoimmune phenomena may develop [11 15]. CVI is probably not a single entity [15]. The proportion of peripheral blood lymphocytes bearing surface Ig varies widely [7 13] and the presence of pre-B lymphocytes does not seem to be a constant finding in the bone marrow [16 20]. Culture of lymphocytes of patients with CVI with PWM leads to variable Ig production and secretion [5 9 26]. There is no uniform extrinsic modulation of B cell function in patients with CVI by T suppressor or helper cells [25] or by serum factors [14].

In this report we present the immunological profile of two immunodeficient patients one of them met the conventional criteria of the WAS, while the other was a case of CVI [12]. The results obtained showed similar disturbances of lymphocytes, which may be involved in the pathogenesis of these diseases.

## Materials and Methods

### Clinical Summary / the Cases

The first case was a 20-year-old man who entered the hospital for thrombocytopenia and recurrent infections of the respiratory tract (upper and lower), as well as of the skin since his first years of life. Family history was unremarkable. Clinical examination revealed bleeding from the gums. Liver spleen or lymph nodes were not enlarged, and the skin was unremarkable. Hb was 16 g/dl, WBC was  $3.5 \times 10^9/l$  (57% polymorphs, 29% lymphocytes, 14% monocytes) platelet count was  $10-50 \times 10^9/l$ , clearance of endogenous creatinine was 80 ml/min, serum protein electrophoresis was within normal range and serum complement was 35 U (normal range 35-55 U); lupus and rheumatoid arthritis tests were negative. X-ray studies revealed chronic sinusitis only.

The second case was a 32-year-old woman with negative family history who entered the hospital for low abdominal pain and fever. She complained also of recurrent respiratory infections during the last 10 years. On examination the spleen was palpable 3 cm below the left costal margin. Lymph nodes, up to 2 cm in diameter were palpated in the axillae and groins. The lower abdomen was tender and on vaginal examination the salpinges were also found to be tender. Hb was 12 g/dl, WBC was  $7.2 \times 10^9/l$  (57% polymorphs, 40% lymphocytes and 3% eosinophils) serum proteins were 5.7 g/dl (albumin 4.3 g, globulin 1.4 g), serum  $\gamma$ -globulin was 0.07 g/dl. On further clinical investigation acute salpingitis was diagnosed and proper therapy was given.

### Isolation of Lymphocytes

Venous blood from patients and healthy controls was drawn into heparin. Mononuclear

cells were isolated on Lymphoprep (Nyegaard Co Oslo, Norway) according to the manufacturer's instructions.

### Rosette Assay

Spontaneous rosettes formed by human lymphocytes with unsensitized sheep erythrocytes, IgG-coated ox erythrocytes and IgG complement coated sheep erythrocytes were formed as described elsewhere [18]. Normal human serum was used as source of complement.

### Detection / Surface Immunoglobulins

Staining for surface immunoglobulins was performed as described previously [19]. All the FITC-conjugated antisera used in this study were obtained from Burroughs Wellcome. The antisera were centrifuged at 45,000 rpm for 90 min before use, to remove aggregated IgG. This way we minimised the interaction of rabbit antihuman immunoglobulins with cells bearing the Fc receptor; this has been proved by using rabbit IgG conjugated with FITC as control.

### Antigen Stimulation

PHA (Burroughs Wellcome) Con-A (Pharmacia) and PWM (Gibco, Lamb) were used as mitogens. Cultures were conducted for 72 h, 6 h before cell harvesting. 0.08  $\mu$ Ci tritiated thymidine was added to each tube culture. The details of the technique have been described elsewhere [19].

### Measurement / Serum Immunoglobulins

For the estimation of the serum immunoglobulins kits of Meloy were used according to the manufacturer's instructions.

## Results

The clinical findings and the low serum IgM (table I) of the first case fulfilled the criteria of the WAS [12]. The clinical picture and the almost complete absence of serum immunoglobulins (table I) led to the diagnosis of common variable immunodeficiency in the second case as shown in table II. E rosettes were high in this patient. The percentage of peripheral lymphocytes



Table I. Serum immunoglobulins

Immunoglobulin	Patients <sup>1</sup>		Normal range
	WAS	CVI	
IgG	1,250 $\pm$ 245	100 $\pm$ 17	1,240 $\pm$ 350
IgA	135 $\pm$ 15	0	250 $\pm$ 80
IgM	33 $\pm$ 4	0	120 $\pm$ 50

<sup>1</sup> The figures (mg/dl) represent the means of 3 independent measurements.

Table II. Surface markers of peripheral lymphocytes

Marker	Patients <sup>1</sup>		Normal range <sup>2</sup>
	WAS	CVI	
E-rosettes, %	56 $\pm$ 7	90 $\pm$ 5	65 $\pm$ 15
EAC rosettes, %	68 $\pm$ 4	60 $\pm$ 6	20 $\pm$ 10
EA-rosettes, %	13 $\pm$ 2	16 $\pm$ 3	20 $\pm$ 15
Surface Ig, %	12 $\pm$ 1.5	4.5 $\pm$ 0.5	10-20
Surface IgM, %	-	1	-
Surface IgG, %	-	3.5 $\pm$ 1	-

<sup>1</sup> The figures are the means of 3 experiments each done in triplicate.

<sup>2</sup> Normal range was obtained from a series of healthy blood donors tested in our laboratory. The controls tested along with the patients were within the same range.

Table III. Blast transformation of peripheral lymphocytes

Mitogen	Patients <sup>1</sup>		Control <sup>2</sup>
	WAS	CVI	
PHA, 10 $\mu$ l	980 $\pm$ 80++	4 136 $\pm$ 300	13,224 $\pm$ 1,505
Con-A			
20 $\mu$ g	1 120 $\pm$ 65	3,249 $\pm$ 820	13 122 $\pm$ 1,045
40 $\mu$ g	1,930 $\pm$ 160	3,068 $\pm$ 181	14 456 $\pm$ 1 756
PWM, 10 $\mu$ l	630 $\pm$ 35	-	9,255 $\pm$ 1,220
No mitogen	82 $\pm$ 15	115 $\pm$ 15	316 $\pm$ 43

<sup>1</sup> The figures represent counts per minute and are the means  $\pm$  1 SD done in triplicate.

<sup>2</sup> Normal healthy blood donor tested along with the patients.

which bore surface Ig were normal in the patient with WAS and very low in the patient with CVI (table II). EA rosettes were normal, whereas EAC rosettes were high in both patients (table II). The tubes, where EAC rosettes were formed, were strongly shaken in a Vortex before counting. By this process spontaneous rosette formation between the sheep erythrocytes and lymphocytes was avoided. Furthermore, lymphocytes incubated with IgG-coated sheep erythrocytes in the same way (as done for EAC rosettes), served as control. Considering together the percentages for E and EAC rosettes, it is evident that a minimum percentage of 24% in the patient with WAS and of 50% in the patient with CVI, simultaneously have receptors for sheep erythrocytes and/or complement.

Peripheral lymphocytes of both patients had a very low capacity to undergo blast transformation with several polyclonal mitogens (table III).

## Discussion

The present study demonstrates, at a cellular level certain similarities between immunological defects, which have quite different manifestations at a clinical or functional level [12]. The 2 patients reported here had low responsiveness to polyclonal mitogens and a major peripheral cell subpopulation bearing T and B characteristics simultaneously.

Blast transformation of peripheral blood lymphocytes from immunodeficient patients is apparently quite variable. Thus, *Blaese et al.* [3, 4] have reported a normal responsiveness in patients with WAS. *Shiff et al.* [4] have reported that the responsiveness

of peripheral lymphocytes of patients with CVI was also normal, whereas others [16, 25] have observed a great variability even within groups in several immunodeficient states. The low responsiveness of peripheral blood lymphocytes (PBL) of our patients may be due to several causes. One possibility is that the subpopulation of PBL demonstrating mitogen responsiveness is lacking; a second possibility is the existence of a subpopulation of PBL which has a suppressor activity on blast transformation as well as on other immunological functions [10, 26]. Finally T lymphocytes from both patients may have not acquired yet as the ability to respond to polyclonal mitogens, thus representing a class of immature cells.

Normally the proportion of human PBL which bear both T and B characteristics is very small (range 1-6%) [1, 8, 21]. The nature of these cells is mostly unknown. Coexistence of receptors for sheep red blood cells and for complement has been reported in 1 patient with chronic lymphatic leukemia [23]. In another patient with a lymphoproliferative disorder [22] and in a certain number of patients with acute lymphoblastic leukemia [2, 17] *Dickler et al.* [7] have reported 1 patient with CVI in whom 20% of the PBL bore receptors for both sheep erythrocytes and surface immunoglobulins.

In conclusion, we demonstrated in both our patients, a subpopulation of lymphocytes which simultaneously bore receptors for sheep erythrocytes and complement and which is unable to respond to polyclonal mitogens such as PHA, Con-A and PWM. Similar findings on lymphoproliferative disorders suggest that such a subpopulation might represent immature T lymphocytes [2, 17]. Although the exact role of this subpopulation of lymphocytes in the expression of

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Normally the proportion of human PBL which bear both T and B characteristics is very small (range 1-6%) [1, 8, 21]. The nature of these cells is mostly unknown. Coexistence of receptors for sheep red blood cells and for complement has been reported in 1 patient with chronic lymphatic leukemia [23] in another patient with a lymphoproliferative disorder [22] and in a certain number of patients with acute lymphoblastic leukemia [2, 17]. *Dickler et al.* [7] have reported 1 patient with CVI, in whom 20% of the PBL bore receptors for both sheep erythrocytes and surface immunoglobulins.

In conclusion, we demonstrated in both our patients, a subpopulation of lymphocytes which simultaneously bore receptors for sheep erythrocytes and complement and which is unable to respond to polyclonal mitogens such as PHA, Con-A and PWM. Similar findings on lymphoproliferative disorders suggest that such a subpopulation might represent immature T lymphocytes [2, 17]. Although the exact role of this subpopulation of lymphocytes in the expression of

the immunological defects is not yet known, it opens new insights into the cellular disturbances of these patients. Further studies, however, are needed to confirm our results in other immunological deficiencies and more sophisticated methods should be applied to define the cellular defects in these patients exactly.

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## Hemoglobin Chain Recombination and the Pattern of Composition of Hemoglobin S and D in Heterozygotes

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**Key Words.** Hemoglobin chain recombination S/D heterozygotes

**Abstract.** The pattern of composition of hemoglobin in different heterozygotes for S and D hemoglobins is quantitatively accounted for by the relative rates of combination of the different chains

$$\alpha + \beta^A > \alpha + \beta^D > \alpha + \beta^S > \alpha + \gamma$$

It is suggested that the recombination step may be one of the rate-limiting steps in hemoglobin assembly

With very few exceptions, all known human hemoglobins are tetramers of two non-identical polypeptide chains which are the primary products of different genes. The synthesis of the chains is independent, and the process of the combination of the chains into tetramers occurs as one of the last steps in the formation of hemoglobins, presumably after the chains have been released from the specific messenger RNA on which they are synthesized. A large number of structural variants of normal adult hemoglobin, which are now known, are the results of mutations of the genes for the different hemoglobin chains. Heterozygotes for such variant hemoglobins generally have two major hemoglobins. It is well known that in heterozygotes for hemoglobin variants the

two alleles function within the same cell. The most direct evidence is the fact that all cells from A/S heterozygotes can be made to sickle under appropriate conditions. For example, individuals with sickle cell trait may have both hemoglobin S and normal hemoglobin in their peripheral blood or hemoglobin S and another variant hemoglobin like HbC or HbD. In the double heterozygote we infer that in each cell at the time of hemoglobin synthesis, there would be simultaneous production of a total of four globin chains, two types of  $\alpha$ -chains and two types of  $\beta$ -chains, and in practically all of these heterozygotes the amount of variant hemoglobin is less than that of the normal hemoglobin. An immediate question which arises is whether these chains will associate in

random or whether an  $\alpha$ -chain will seek preferential association with one of the two different  $\beta$ -chain partners available. The factors that determine the concentration of the different hemoglobins in the heterozygotes are not fully known. Neel and co-workers [13] have suggested that genetic rather than environmental causes may be responsible for the variation in blood hemoglobin ratios since in a heterozygote for a  $\beta$ -chain variant, who also inherits simultaneously an  $\alpha$ -thalassemia gene, the proportion of the variant hemoglobin is much lower than in simple heterozygotes [5].

The mechanism whereby the 1:1 stoichiometry between  $\alpha$ - and  $\beta$ -chains is ensured in red cells is still unknown. It is, however well established that in heterozygotes for hemoglobin abnormalities the ratio of the two hemoglobin species present often deviates from unity. The theoretical explanation that has been proposed was that of preferential subunit association. For instance, in the case of A/S heterozygotes, one might postulate a preference of the  $\alpha$ -chain for  $\beta^A$  compared to  $\beta^S$ . If the production rates of the two  $\beta$ -chains are equal, this would, of course, imply that a certain proportion of  $\beta^S$ -chains synthesized are never incorporated into tetramers and are perhaps disposed of during red cell maturation. The significant role of the rate of association of the different chains in the determination of the pattern of composition of hemoglobin in sickle cell heterozygotes is not known and has not been previously investigated.

In this report we have therefore measured the rates of recombination of the different chains in order to evaluate if the rate of recombination of the hemoglobin chains could possibly be the rate-limiting step in the production of intact hemoglobins and

might be the factor that determines the pattern of composition of hemoglobins in heterozygotes.

### Material and Methods

Blood samples were collected in anticoagulant, acid-citrate dextrose (ACD) or heparin and used immediately.

#### *Concentration of Reticulocytes*

Blood, collected in ACD, was spun at 8,000 rpm for 5 min in a Sorvall refrigerated centrifuge at 4°C to remove plasma. Concentrated cells were spun again at 20,000 rpm for 50 min. The top 0.5 ml of each centrifuge portion was removed as reticulocytes. The reticulocytes were then pooled and resuspended in minimum amount of normal saline solution. This procedure served to increase the reticulocyte percentage.

#### *Preparation and Characterization of Hemoglobin Types*

Hemoglobin solutions from red blood cells and from reticulocytes were prepared by the usual method of washing with normal saline and then lysing the blood with 2 vol water. Initial identification of hemoglobin types, was made by cellulose acetate electrophoresis in Tris-EDTA-borate buff er pH 8.6.

The acid elution technique was used to screen for stainable fetal hemoglobin [11], and the percentage of fetal hemoglobin in the hemolysate of patient known to be heterozygous for HbS and HbF was determined according to the method of Huxson and Meyering [9].

#### *Preparation and Kinetics of Recombination of Hemoglobin Chains*

The different polypeptide chains in normal, fetal, and sickle cell hemoglobins were prepared by the methods of Bucci and Fronticelli [3] with minor modifications. The perchloromercuribenzoate (PCMB) bound to the sulfhydryl groups of the chains was removed by washing with mercaptoethanol. The hemoglobin chains which have been freed of mercaptoethanol were used for kinetic recombination experiments after deoxygenation



with dithionite. It has been reported by Antonini *et al.* [1] that, at 430 nm, the deoxygenated chains have lower extinction coefficients than intact hemoglobins. Thus, the rate of combination of the different chains into tetrameric whole hemoglobins was followed by the change in optical absorption at 430 nm. The chains were loaded into stock syringes of a Gibson Durrum stopped flow spectrophotometer. Rapid mixing of the different chains was effected by the method of Gibson and Miles, as previously described [1] and the change in optical density with time was monitored on an oscilloscope. The record of the oscilloscope trace is shown in Figure 1.

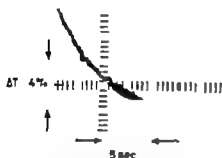


Fig. 1 Stopped flow oscilloscope trace of percentage transmittance ( $\Delta T$ ) versus time in seconds for the rate of combination of  $\alpha$ -chains with  $\beta^S$ -chains. The experimental details are described in the text.

Table I. Half-life time  $T_{1/2}$  (sec), mean  $\pm$  SD for recombination of hemoglobin chains at 430 nm

	$\alpha$	$\beta$	$\beta^S$	$\gamma$
$\alpha$	-	$2.8 \pm 0.2$	$4.8 \pm 0.2$	$31.5 \pm 0.5$

Table II. Second-order rate constants ( $M^{-1} \text{ sec}^{-1}$ ), mean  $\pm$  SD for the recombination of hemoglobin chains at 430 nm

	$\alpha$	$\beta$	$\beta^S$	$\gamma$
$\alpha$	-	$(9.0 \pm 0.1) \times 10^4$	$(5.2 \pm 0.3) \times 10^5$	$(0.78 \pm 0.1) \times 10^6$

### Solubility and Purification of Hemoglobin D

Solubility measurements were carried out according to the modified method of Itano [10].

Hemoglobin D Ibadan was purified from the blood sample from the propositus, who is heterozygous for hemoglobins D and S, by the solubility method of Itano [10] according to the modification of Watson Williams *et al.* [15] and also by a pH gradient chromatography on carboxymethyl cellulose of the hemoglobin of an A+D heterozygote, using a modified method of Huisman and co-workers [8].

### Results and Discussion

Figure 1 shows the trace of a typical chain recombination reaction between  $\alpha$ - and  $\beta^S$ -chains. The half-life (i.e., the time for 50% formation of intact hemoglobin from the chains) and the rate constant of the chain combination reaction were determined from the oscilloscope trace. These results are shown in tables I and II. The results gave the increasing rate of combination as:

$$\alpha + \beta^1 > \alpha + \beta^S > \alpha + \gamma$$

This result thus shows that  $\alpha$ -chains combine at faster rate with  $\beta^1$ -chains from normal adult than with  $\beta^S$ -chains from sickle cell hemoglobin and at a more than 10 times faster rate with  $\gamma$ -chains from fetal hemoglobin. The results in tables I and II would therefore predict preferential association of the chains in such a way that in the presence of saturating amounts of  $\alpha$ -  $\beta^1$ - and  $\beta^S$ -chains there would be a greater per

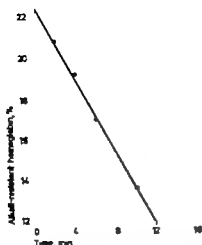


Fig. 2. Plot of the percentage alkali-resistant hemoglobin remaining in solution after alkaline denaturation for different time intervals for the hemolysate from patient heterozygous for sickle and fetal hemoglobins. The percentage of fetal hemoglobin is obtained by extrapolation of the denaturation curve to zero time.

centage of HbA than of HbS. Similarly with saturating amounts of  $\alpha$ - $\beta^L$ - and  $\gamma$ -chains the percentage of HbS would be higher than that of HbF and with saturating amounts of  $\alpha$ - $\beta^L$ - and  $\gamma$ -chains the percentage of HbA would be higher than that of HbF.

Figure 2 shows the plot of percentage of the alkali-resistant hemoglobin remaining after denaturation of the hemoglobin sample with alkali for different times. The hemoglobin sample was from the hemolysate of a patient characterized by both starch gel and cellulose acetate electrophoresis to have hereditary persistence of fetal hemoglobin and to be heterozygous only for hemoglobins S and F. The percentage of hemoglobin F obtained at zero time of the alkaline denaturation curve in figure 2 is 22% and

hence, by difference, the percentage composition of hemoglobin S in the hemolysate would be 78%. The percentage compositions are further confirmed by the analysis of the chromatographic elution peak for hemoglobin S and F in the chromatographic separation of the hemolysate from the same patient, on Amberlite Resin CG 50 by the method of Huisman *et al.* [9] using citrate buffer pH 6.0. Thus we found that in a patient who is heterozygous for hemoglobins S and F the pattern of hemoglobin composition is HbS > HbF.

Chromatographic separation on carboxy methyl cellulose of hemolysate from a patient who is heterozygous for hemoglobins A and D by the method of Huisman *et al.* [8] revealed that hemoglobin D is 41%. This is confirmed by the quantitative estimation of the eluted hemoglobins A and D from the cellulose acetate electrophoresis. Thus in an A/D heterozygote the hemoglobin pattern of composition is HbA > HbD.

Hemoglobins S and D from a patient who is an S/D heterozygote could not be separated chromatographically. The composition of hemoglobin D was, however estimated by changes in optical density or residual radioactivity after precipitation of hemoglobin S from the hemolysate of the red cell of the radioactively labelled reticulocytes of an S/D heterozygote. The percentage composition of D was found to be 57 and 59%, respectively by the two methods. The result is also in agreement with values obtained from solubility data in figure 3 after applying the solubility relation of composite mixture of hemoglobin as shown by the expression

$$S_{S+D} = S_D + (1-s)S_S$$

where  $S_{S+D}$  is the solubility of the hemolysate from an S+D heterozygote,  $S_D$  and

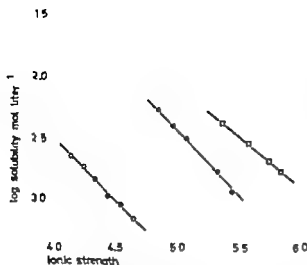


Fig. 3 Plot of log of solubility of deoxy hemoglobins against ionic strength in phosphate buffer pH 7.0, O = Purified hemoglobin S □ = purified hemoglobin D ● = hemolysate from a patient heterozygous for S and D hemoglobins.

$S_2$  are the solubilities of pure hemoglobins D and S respectively and  $\alpha$  is the fraction of hemoglobin D present in the hemolysate. From the graph of figure 3 we were able to obtain the solubilities of pure HbS and of pure HbD at the same ionic strength. From the value of the measured solubility of the hemolysate at the same ionic strength, from the same figure 3 we can then use the above expression to calculate the value of  $\alpha$ , the fraction of HbD present in the hemolysate. The higher percentage of HbD (57%) in a D+S heterozygote obtained by this method indicates that hemoglobin D is formed in preference to HbS in an S+D heterozygote unlike in A+D heterozygotes where hemoglobin D is lower (41%) indicating that HbA is preferentially formed to HbD. Similarly in an S+F heterozygote, the higher percentage of HbS could be attributed to hemoglobin S being formed in preference to hemoglobin F. The relative rate of combination of the different chains

reported in this paper would thus correspond to the observed relative ratio of the hemoglobin composition in these heterozygotes. If the rate of combination of the chains is the rate-limiting step in hemoglobin synthesis, heterogeneity of composition in heterozygotes could therefore be determined at this level. From the percentage composition of hemoglobin D 57 and 41% in S + D and A + D heterozygotes, respectively it could be predicted that an  $\alpha$ -chain would recombine slightly faster with a  $\beta^L$ -chain than with a  $\beta^S$ -chain and thus give rise to the relative recombination rates as:

$$\alpha + \beta^A > \alpha + \beta^D > \alpha + \beta^S > \alpha + \gamma$$

Chain association of normal human adult hemoglobin was studied by Huehns and his colleagues [7] in 1964 using starch block electrophoresis. Their results are similar to ours except that the reaction was still taking place for up to 4 h. This difference could probably be explained by the difference in the techniques used and in the hemoglobin concentrations.

A hemoglobin with an abnormality of the  $\alpha$ -chain and having the electrophoretic mobility of hemoglobin S at pH 8.6 has been found among Nigerian blood donors with a frequency of about 0.4%.

Subjects doubly heterozygous for this abnormal hemoglobin and for hemoglobin S produce the expected four hemoglobin species,

$$\alpha^{\text{normal}}\beta^{\text{normal}}, \alpha^{\text{normal}}\beta^{\text{S}}, \alpha^{\text{abnormal}}\beta^{\text{normal}}, \alpha^{\text{abnormal}}\beta^{\text{S}}$$

In these double heterozygotes, the data of Esan *et al* [unpublished data] on the quantitation of the individual hemoglobin components shown in figure 4 could be explained by our kinetic result and further confirms that association of different hemoglobin chains in the erythrocyte does not

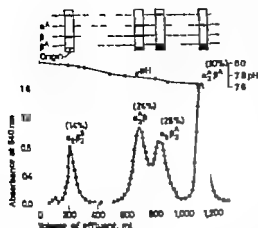


Fig. 4. Resolution of four hemoglobin components from hemolyate of double heterozygotes with  $\alpha$ - and  $\beta$ -chain abnormalities. Column chromatography on DEAE Sephadex was carried out as follows: The technique of Huisman *et al.* [5] with some modifications was used to separate the hemoglobins from patient heterozygotes for HbS and  $\alpha$ -chain abnormality. 200 mg of hemoglobin in 2 ml of hemolysate was applied to  $2.5 \times 90$  cm column, at  $4^\circ\text{C}$ . Chromatographic elution was carried out by means of linear pH gradient obtained by mixing 1 liter of  $0.05\text{ M}$  Tris-HCl buffer pH 7.8, with 1 liter of  $0.05\text{ M}$  Tris-HCl buffer pH 7.0. The sample was dialyzed against the pH 7.8 buffer before application onto the column. 10-ml fractions of the effluent were collected until all hemoglobin was eluted. The optical density of each 10-ml fraction was monitored at 540 nm on Griford 2000 spectrophotometer. Individual globin chains of separated hemoglobins were obtained by electrophoresis fractions in 8 M urea at pH 8.6. The diagram at the top of the figure illustrates the identification of the polypeptide chains present in each of the four peaks eluted from the column.

take place at random. The data in figure 4 in agreement with our kinetic data, therefore demonstrate that the normal chains seem to combine preferentially with the normal rather than with the abnormal hemoglobin chains. Thus our kinetic data support

the notion of preferential association of different  $\beta$ - and  $\gamma$ -chain variants for the same  $\alpha$ -chain.

Even though it has been previously reported by Esau and others [5, 6, 13] that the ratios of hemoglobins in heterozygotes correspond to the relative rates of synthesis of the various chains, our present results show that the combination rate also quantitatively predicts the pattern of distribution of hemoglobins in simple heterozygotes.

The relatively slow combination rate of  $\alpha$ -chains with  $\gamma$ -chains as compared with the combination rate of  $\alpha$ - and  $\beta$ -chains could account for the occurrence of hemoglobin "Barts" in some newborn babies with a relative excess of  $\gamma$ -chains. The excess of  $\gamma$ -chains could have been the result of a reduced  $\alpha$ -chain synthesis, that is,  $\alpha$ -thalassaemia, or due to a developmental abnormality resulting from asynchronism of the switching off from  $\gamma$ - to  $\beta$ -chain synthesis, as has been postulated from data on Nigerian newborn babies [5].

The preferential association of one hemoglobin chain for another could possibly be genetically determined, as intergenetic complementation could take place between products of different gene activities. However the extent to which complementation could occur may depend on the degree of correspondence between different  $\alpha$ - and  $\beta$ -chains. It may be this complementation that has given rise to the expression of the preferential rate of association of the chains in tables I and II thus giving expression to the quantitative pattern of hemoglobin components in heterozygotes as shown in figure 4. Nonrandom association of hemoglobin chains may therefore have some evolutionary significance through the mechanism of the structure of one chain imposing con-

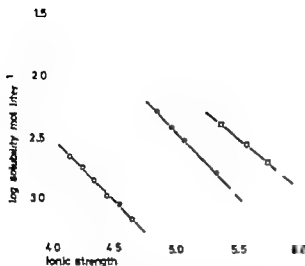


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In these double heterozygotes, the data of Esan *et al* [unpublished data] on the quantitation of the individual hemoglobin components shown in figure 4 could be explained by our kinetic result and further confirms that association of different hemoglobin chains in the erythrocyte does not

## Influence of Mithramycin on Some Platelet Functions *in vitro*

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**Key Words.** Mithramycin. Platelet aggregation. Platelet function. Platelet release

**Abstract.** Platelet rich plasma was incubated with mithramycin *in vitro*. This diminished platelet aggregation by ADP and adrenaline, but did not interfere with collagen-induced aggregation. Platelet factor 4 release was diminished by ADP and delayed when induced by adrenaline but normal when induced by collagen. Platelet factor 3 availability was not significantly impaired. Reptilase clot retraction was diminished when induced by ADP but normal when induced by collagen. Uptake of  $^3\text{H}$ -serotonin and  $^3\text{H}$ -adenine was slightly inhibited. There was no depression in platelet adhesion or release of serum aggregating activity.

### Introduction

Mithramycin is a cytostatic drug that is used for treatment of testicular tumors and hypercalcemia. The most important form of toxicity associated with its use consists of a bleeding syndrome which usually begins with an episode of epistaxis. In some cases it may progress to a widespread hemorrhage in the gastrointestinal tract or to a generalized bleeding tendency. The hemorrhagic syndrome seems to be dose-related. It is associated with multiple abnormalities in hemostasis. Thus, the clotting time is prolonged, the clot retraction diminished, the prothrombin content decreased and the platelet count depressed.

Mithramycin may exert its action on the formation of platelets and coagulation factors, but it may also interfere with the platelets themselves. In the present study its influence on the platelet function was investigated *in vitro* using a wide battery of tests.

### Material and Methods

Blood samples are obtained from healthy volunteers by allowing 9 parts of venous blood to flow directly into test tubes containing 1 part of 0.11 mol trisodium citrate.

Platelet-rich plasma (PRP) was obtained by centrifuging the blood at 280 g for 20 min at 16 °C. The platelet count was adjusted to 300,000  $\mu\text{l}$  by addition of platelet-poor plasma (PPP) from

straints on the mutational changes in the other since the correspondence between the chain partners would be affected. By this mechanism, the amino acid composition of the inter-subunit region would be very critical and would only undergo very selective mutations that preserve the correspondence between the partner chains.

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Table II. Effect of mithramycin on the platelet factor 3 availability: recalcification time in seconds (means  $\pm$  standard deviations of 8 experiments)

		Time of exposure with inducer min		ADP-Induced (4.5 $\mu$ M) availability		Adrenaline-Induced (6 $\mu$ M) availability		Collagen-Induced (20 mg/l) availability	
				Preincubation with mithramycin or buffer		Preincubation with mithramycin or buffer		Preincubation with mithramycin or buffer	
				5 min	30 min	5 min	30 min	5 min	30 min
PRP + buffer	0			35 $\pm$ 2		37 $\pm$ 2		37 $\pm$ 2	
	5			22 $\pm$ 4		23 $\pm$ 4		23 $\pm$ 5	
	10			19 $\pm$ 3		19 $\pm$ 3		19 $\pm$ 3	
	20			18 $\pm$ 2		17 $\pm$ 2		18 $\pm$ 3	
PRP + mithramycin 0.46 $\mu$ M	0			31 $\pm$ 3	33 $\pm$ 2	31 $\pm$ 3	33 $\pm$ 2	31 $\pm$ 3	34 $\pm$ 2
	5			25 $\pm$ 6	27 $\pm$ 4	24 $\pm$ 4	28 $\pm$ 3	24 $\pm$ 6	29 $\pm$ 3
	10			22 $\pm$ 4	26 $\pm$ 3	20 $\pm$ 4	24 $\pm$ 2	21 $\pm$ 5	27 $\pm$ 2
	20			20 $\pm$ 4	22 $\pm$ 2	19 $\pm$ 3	21 $\pm$ 2	19 $\pm$ 4	4 $\pm$ 2
PRP + mithramycin 2.3 $\mu$ M	0			33 $\pm$ 3	33 $\pm$ 2	34 $\pm$ 2	34 $\pm$ 2	32 $\pm$ 2	35 $\pm$ 2
	5			28 $\pm$ 7	27 $\pm$ 5	24 $\pm$ 3	27 $\pm$ 3	24 $\pm$ 5	25 $\pm$ 4
	10			23 $\pm$ 6	23 $\pm$ 5	21 $\pm$ 3	22 $\pm$ 4	21 $\pm$ 4	22 $\pm$ 4
	20			21 $\pm$ 5	21 $\pm$ 4	19 $\pm$ 3	20 $\pm$ 3	19 $\pm$ 4	20 $\pm$ 4

Table III. Percentage platelet factor 4 released after 0.5 and 10 min exposure of PRP to different inducers following incubation for 5 and 30 min with mithramycin in concentrations of 0.46 and 2.3  $\mu$ M (means  $\pm$  SD of 9 experiments)

		Time of exposure with inducer min		Release induced by ADP (4.5 $\mu$ M)		Release induced by adrenaline (6 $\mu$ M)		Release induced by collagen (20 mg/l)	
				Preincubation with mithramycin or buffer		Preincubation with mithramycin or buffer		Preincubation with mithramycin or buffer	
				5 min	30 min	5 min	30 min	5 min	30 min
PRP + buffer	0			7 $\pm$ 2		7 $\pm$ 3		7 $\pm$ 1	
	5			40 $\pm$ 3		43 $\pm$ 4		47 $\pm$ 6	
	10			46 $\pm$ 4		49 $\pm$ 3		54 $\pm$ 3	
PRP + mithramycin 0.46 $\mu$ M	0			7 $\pm$ 3	7 $\pm$ 2	7 $\pm$ 3	7 $\pm$ 3	7 $\pm$ 3	7 $\pm$ 3
	5			27 $\pm$ 9	18 $\pm$ 6	42 $\pm$ 5	18 $\pm$ 10	48 $\pm$ 4	47 $\pm$ 7
	10			30 $\pm$ 3	15 $\pm$ 5	50 $\pm$ 3	39 $\pm$ 13	53 $\pm$ 3	49 $\pm$ 6
PRP + mithramycin 2.3 $\mu$ M	0			7 $\pm$ 2	7 $\pm$ 3	7 $\pm$ 2	7 $\pm$ 3	7 $\pm$ 1	7 $\pm$ 3
	5			29 $\pm$ 10	12 $\pm$ 4	43 $\pm$ 4	17 $\pm$ 10	43 $\pm$ 7	49 $\pm$ 4
	10			37 $\pm$ 9	18 $\pm$ 8	50 $\pm$ 4	44 $\pm$ 3	52 $\pm$ 4	57 $\pm$ 6



the same volunteer PPP was prepared by centrifuging PRP at 12,000 g for 20 min at 17 °C.

ADP (Sigma) collagen (Stago Asnières, France), and adrenaline (Spofa Prague, Czechoslovakia) were used and diluted to the desired concentrations by addition of Michaelis's buffer at pH 7.35.

Mithramycin (Mithracin Pfizer New York, N.Y.) was dissolved to the desired concentration in sterile water. In most investigations mithramycin in concentrations of 0.46 or 2.3  $\mu$ M was incubated with PRP for 5 or 30 min in a water bath at 37 °C.

Platelet aggregation was studied measuring the change in optical density of PRP [1] in a platelet aggregometer (Evans Electroscintum Ltd.), connected to a pen recorder to permit the automatic registration in transmitted light. Sensitivity was adjusted so that PPP set close to 100% and PRP to 0%. Reagents were added to the cuvette of the aggregometer as follows: 0.9 ml of PRP, 0.1 ml of buffer with mithramycin, and 0.1 ml of the inducer of aggregation. Platelet aggregation was performed at 37 °C with automatic stirring.

The availability of platelet factor 3 and release of platelet factor 4 (PF 4) were estimated by methods described in details elsewhere [5].

The adhesion of platelets to glass slide was assessed by using the method of Mason and Gilkey [7] as modified by Kordová and Kubitz [4].

The release of serum aggregating activity was measured according to Caen *et al* [1].

Reptilase clot retraction was measured as described previously [6] using Reptilase (Pentapharm, Basel, Switzerland, or Stago, Asnières, France).

Uptake of  $^3$ H-serotonin was measured as follows: PRP (0.8 ml) was preincubated for 5 or 30 min at 37 °C with 0.1 ml buffer or mithramycin solution. Afterwards, 0.7  $\mu$ M final concentration of  $^3$ H-serotonin (Radiochemical Center, Amersham, England) was added and PRP was incubated for an additional 30 min. Serotonin was dissolved in 12 M ethanol, to a concentration of 8  $\mu$ Cl/ml and 0.14 mM. The samples were gently mixed without stirring or shaking during the incubation period. They were then cooled in an ice bath and subsequently centrifuged at 4 °C for 5 min at 7,000 g and supernatant plasma was collected. Radioactivity was counted for 1 min in a liquid scintillation counter (Intertechnique, France) after addition of uncentrifuged PRP or supernatant (0.1 ml) to a dioxane-naphthalene scintillation mixture. Uptake of serotonin was calculated by the following formula.

Uptake % =

$$\frac{\text{counts min}^{-1}(\text{PRP}) - \text{counts min}^{-1}(\text{supernatant})}{\text{counts min}^{-1}(\text{PRP})}$$

Adenine uptake was measured according to Dantel *et al* [2].  $^3$ H-adenine (Amersham) with a specific activity of 21 Ci/mmol was used in a final concentration of 0.1  $\mu$ Cl/ml PRP.

Table L. Effect of mithramycin on platelet aggregation induced by ADP, adrenaline and collagen (means of 5 experiments)<sup>1</sup>

	ADP-induced aggregation		Adrenaline-induced aggregation		Collagen-induced aggregation	
	Preincubation with mithramycin or buffer		Preincubation with mithramycin or buffer		Preincubation with mithramycin or buffer	
	5 min	30 min	5 min	30 min	5 min	30 min
PRP + buffer	80		84		89	
PRP + mithramycin 0.46 $\mu$ M	62	68	66	76	81	84
PRP + mithramycin 2.3 $\mu$ M	49	38	56	55	78	77

<sup>1</sup> Platelet aggregation was induced by ADP (4.5  $\mu$ M), adrenaline (6  $\mu$ M) or collagen (20 mg/l). Data are given as percent increase in light transmission after 4 min.

Table VI. Uptake of  $^{14}\text{C}$ -serotonin and  $^{14}\text{C}$ -adenine (means  $\pm$  SD), %

	Uptake of serotonin Preincubation with mithramycin or buffer		Uptake of adenine Preincubation with mithramycin or buffer	
	5 min	30 min	5 min	30 min
PRP + buffer	88 $\pm$ 4		40 $\pm$ 5	
PRP + mithramycin 0.46 $\mu\text{M}$	86 $\pm$ 4	80 $\pm$ 5	38 $\pm$ 5	32 $\pm$ 6
PRP + mithramycin 2.3 $\mu\text{M}$	82 $\pm$ 5	78 $\pm$ 5	32 $\pm$ 6	30 $\pm$ 6

Table VII. Stabilizing effect (%) on platelet membranes measured as the increase in light transmission (following repeated freezing and defreezing 1-6 times (means  $\pm$  SD of 6 experiments))

	1 freezing Preincubation with mithramycin or buffer		2 freezings Preincubation with mithramycin or buffer		4 freezings Preincubation with mithramycin or buffer		6 freezings Preincubation with mithramycin or buffer	
	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min
PRP + buffer	13 $\pm$ 5		19 $\pm$ 6		25 $\pm$ 7		32 $\pm$ 5	
PRP + mithramycin 0.46 $\mu\text{M}$	14 $\pm$ 5	14 $\pm$ 6	21 $\pm$ 6	19 $\pm$ 7	28 $\pm$ 7	23 $\pm$ 7	33 $\pm$ 5	39 $\pm$ 6
PRP + mithramycin 2.3 $\mu\text{M}$	13 $\pm$ 6	14 $\pm$ 6	19 $\pm$ 7	21 $\pm$ 7	27 $\pm$ 6	25 $\pm$ 7	33 $\pm$ 4	31 $\pm$ 5

Table VIII. Effect of mithramycin on some coagulation parameters (means  $\pm$  SD of 6 experiments)

	Quick time (sec) Preincubation with mithramycin or buffer		Cephalin-thrombin time (sec) Preincubation with mithramycin or buffer		Reptilase time (sec) Preincubation with mithramycin or buffer		Thrombin time (sec) Preincubation with mithramycin or buffer	
	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min
PRP + buffer	13.2 $\pm$ 0.9		40.6 $\pm$ 2.2		25.5 $\pm$ 3.0		27.3 $\pm$ 1.8	
PRP + mithramycin 0.46 $\mu\text{M}$	12.8 $\pm$ 1.1	13.1 $\pm$ 1.0	39.4 $\pm$ 2.1	39.9 $\pm$ 2.5	25.1 $\pm$ 2.8	25.2 $\pm$ 3.2	26.9 $\pm$ 1.6	26.8 $\pm$ 1.5
PRP + mithramycin 2.3 $\mu\text{M}$	12.5 $\pm$ 1.2	12.7 $\pm$ 0.9	39.7 $\pm$ 1.9	40.3 $\pm$ 2.0	24.8 $\pm$ 3.3	25.0 $\pm$ 3.1	27.2 $\pm$ 1.6	26.3 $\pm$ 2.1

Table IV Effect of mithramycin on reptilase clot retraction, %<sup>1</sup>

	Clot retraction time, min	ADP-induced retraction Preincubation with mithramycin or buffer		Collagen-induced retraction Preincubation with mithramycin or buffer	
		5 min	30 min	5 min	30 min
PRP + buffer	15				
	30	36 ± 9		27 ± 7	
	45	62 ± 10		55 ± 9	
PRP + mithramycin 0.46 $\mu$ M	15	70 ± 8		83 ± 10	
	30	28 ± 7	25 ± 8	15 ± 7	12 ± 8
	45	55 ± 11	42 ± 12	60 ± 8	48 ± 9
PRP + mithramycin 2.3 $\mu$ M	15	68 ± 9	54 ± 10	80 ± 10	78 ± 11
	30	35 ± 10	25 ± 8	23 ± 8	15 ± 6
	45	63 ± 11	40 ± 9	56 ± 11	47 ± 10
		72 ± 8	55 ± 9	79 ± 11	81 ± 10

<sup>1</sup> PRP was incubated with buffer or mithramycin for 5 and 30 min at 37°C. The platelets were activated by ADP (0.1 mM) or collagen (50 mg/l). Immediately afterwards reptilase was added, and the volume of fluid separated from the clot after 15, 30 and 45 min was expressed as a percentage of total (means ± SD of 8 experiments).

Table V Effect of mithramycin on platelet adhesion (means ± SD of 6 experiments), arbitrary units

	Adhesion to glass slide Preincubation with mithramycin or buffer	
	5 min	30 min
PRP + buffer	29 ± 3	
PRP + mithramycin 0.46 $\mu$ M	24 ± 5	24 ± 4
PRP + mithramycin 2.3 $\mu$ M	24 ± 4	26 ± 5

Stability of platelet membranes was tested by measuring the increase in light transmission on repeated freezing and thawing as described by de Gaetano et al. [3].

The Quick time, cephalin-kaolin time, reptilase time, thrombin time and fibrinolytic activity in euglobulin clots were determined according to Nilsson [8].

## Results

Aggregation induced by ADP or adrenaline was diminished by preincubation with mithramycin (table I). This inhibition was more pronounced when mithramycin was added at a concentration of 2.3  $\mu$ M than at 0.46  $\mu$ M. Collagen induced aggregation was not impaired.

Platelet factor 3 release was but little influenced (table II). Platelet factor 4 release was diminished when induced by ADP and delayed when induced by adrenaline (table III). It was not influenced when induced by collagen. The inhibition was more pronounced after prolonged preincubation with mithramycin for 30 min than for 5 min, irrespective of dosage.

Reptilase clot retraction was slightly depressed when induced by ADP but only after

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**Key Words.** Fitzgerald factor    Fitzgerald trait    Fletcher factor    High-molecular-weight kininogen    Plasma prekallikrein

**Abstract.** Asymptomatic, female, 56-year-old identical Japanese twins were found to have a severe abnormality in the surface-mediated intrinsic coagulation, fibrinolysis and esterolytic activity. These defects were thought to be due to the lack of Fitzgerald factor because of the prolongations of kaolin-activated partial thromboplastin time and kaolin-activated euglobulin lysis time that were not corrected by the addition of Fitzgerald trait plasma but were corrected to normal levels by the addition of isolated bovine high-molecular weight kininogen, Fletcher trait-plasma or Hageman trait-plasma.

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follow the activation effects of Hageman and plasma prekallikrein in the generation of clot-promoting activity [9]. HMW-kininogen also facilitates activation of Hageman factor [10].

This paper reports the first cases of Fitzgerald factor deficiency: 3 cases in one family in the Orient, who have low levels of plasma prekallikrein.

### Materials and Methods

#### *Case Report*

The proband (case 1) is a 56-year-old identical Japanese female twin without previous hemorrhagic tendencies. She was referred to the Okayama University Hospital because of prolonged

30 min incubation with mithramycin. It was normal when induced by collagen (table IV)

Platelet adhesion was normal (table V)  
Uptake of  $^{14}\text{C}$  serotonin and  $^{14}\text{C}$ -adenine was slightly inhibited at 30 min (table VI)

There was no interference with the stability of platelet membranes, as manifested by increase in light transmission following repeated freezing and thawing (table VII)

The coagulation factors were not influenced *in vitro* (table VIII) The euglobulin clot lysis time always exceeded 3 h indicating absence of increased fibrinolytic activity

## Discussion

The concentrations of mithramycin used were chosen so that the lower dose (0.46  $\mu\text{M}$ ) corresponded to concentrations met in plasma in patients treated with the drug.

The investigations revealed impaired aggregation of platelets following the addition of the drug *in vitro*. This was consistent with a diminished release of platelet factor 4 and decreased reptilase clot retraction. It was interesting to see that the impairment of platelet factor 4 release was more marked following preincubation for 30 min than for 5 min irrespective of concentration. No such influence was found in the controls. It indicates that the drug may interfere with some receptor inside the platelets.

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The abnormality found was mild, but it cannot be excluded that it may contribute to the bleeding tendency sometimes observed *in vivo*

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The abnormality found was mild, but it cannot be excluded that it may contribute to the bleeding tendency sometimes observed *in vivo*.

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Table I. Routine coagulation studies

	Control	Case 1	Case 2	Case 3
Recalcification time, min	2.0	> 10	ND	ND
Prothrombin time, sec	12.6	13.0	11.6	12.8
APTT <sup>a</sup> with ellagic acid, sec	31.0	243.4	209.0	252.2
APTT with kaolin, sec	42.2	628.0	434.6	526.0
Thrombin time, sec	13.2	11.6	13.8	12.4
Serial thrombin time, sec	17.4	16.0	17.6	16.2
Fibrinogen, mg/dl	354.0	330.0	292.0	ND
Cryofibrinogen, mg/dl	negative	2.6	negative	negative
FDP <sup>b</sup> $\mu$ g/mg	negative	negative	negative	negative
Euglobulin lysis time, h	7.0	16.5	ND	ND

Identical twins.

ND = Not determined.

<sup>a</sup> Activated partial thromboplastin time.

<sup>b</sup> Fibrin degradation product.

Table II. Cross-correction studies using APTT<sup>a</sup> (sec) of patient plasma (PT-PL) with the addition of other deficient plasma

	Case 1	Case 2	Case 3
PT-PL	261.8	290.0	255.2
PT-PL + Factor VIII-deficient plasma (3:2)	30.8	31.4	35.0
PT-PL + Factor IX-deficient plasma (3:2)	40.2	29.0	31.0
PT-PL + Factor XI-deficient plasma (3:2)	39.6	36.0	40.4
PT-PL + Factor XII-deficient plasma (3:2)	31.6	30.2	28.0
PT-PL + Fletcher trait-plasma (3:2)	37.2	32.0	34.4
PT-PL + Fitzgerald trait-plasma (3:2)	256.0	311.4	260.2

APTT was measured using ellagic acid as the activating agent with a 2-min incubation period.

corrected almost to normal by the addition of normal serum or BaSO<sub>4</sub>-adsorbed normal plasma. The prolonged APTT was not corrected by the addition of Fitzgerald trait plasma to the patient plasma but was completely corrected by the addition of Factor XI-deficient plasma, XII-deficient plasma or Fletcher trait-plasma (table II). The abnormal APTT was only partially corrected after prolonged incubation with kaolin or ellagic acid.

In procoagulant assays for clotting factors, the content of Fitzgerald factor was less than 1% of the normal plasma which was estimated by APTT. The other factors were present at normal levels except Fletcher factor. Fletcher factor was present at a concentration of 28% of normal. Factor XI was 88% of normal by the one-stage method with congenital human deficient plasma, whereas the same factor was 28-40% of normal by the same method in which bovine



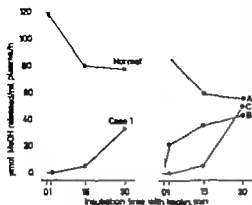


Fig. 2. Kaolin-activated TAME esterase activity of patient plasma (case 1) and its correction upon mixing with other deficient plasmas. The kaolin-activated plasma esterase activity for TAME was assayed by the method of Coburn *et al.* [6]. A = Case 1 + normal (1:1); B = case 1 + Fletcher trait (1:1); C = case 1 + Fitzgerald trait (1:1). MeOH = Methanol.

Another twin (case 2) and her elder sister (case 3) had the same defect of surface-mediated intrinsic clotting, fibrinolysis and esterolytic activity due to HMW kininogen deficiency. The daughter of the proband (case 1) was also partially defective in Fitzgerald factor clotting activity (fig. 1).

## Discussion

The coagulation-factor deficiency due to the lack of HMW-kininogen has been reported in only 5 families in the literature [2, 6, 8, 9, 18]. In this deficiency like in Hageman factor and prekallikrein-deficiency there are profound abnormalities in *in vitro* surface-mediated coagulation, fibrinolysis and kinin generation, yet there is no clinical evidence of bleeding.

We have reported the first cases in the Orient of this deficiency which were found in 56-year-old identical Japanese twins and their elder sister. Our case also showed abnormalities in surface-mediated coagulation, fibrinolysis and esterolytic activity with low levels of plasma prekallikrein. Partial deficiency of prekallikrein activity in kininogen deficiency has been reported in patients with Fitzgerald trait [2, 9], Williams trait [8] and Reid trait [18] and could be explained in several ways [8, 10, 19]. On the one hand, the deficiencies in prekallikrein and HMW-kininogen may reflect a genetically determined defect of synthesis, on the other hand, the deficiency in prekallikrein may be due to a hypercatabolism which could be a consequence of a deficiency in HMW-kininogen, owing to the fact that it exists as a complex with prekallikrein in the plasma and thus stabilizes prekallikrein [10].

The procoagulant activity of Factor XI assayed by the one-stage method within human deficient plasma was 88% of normal, but the same factor estimated by using bovine deficient plasma was 28–40%. This discrepancy may be due to species incompatibility between human plasma and bovine plasma for activation of Factor XI.

The kaolin-activated arginine esterolytic activity was markedly decreased but was enhanced by prolonged plasma activation with kaolin such as exists in Williams trait and Reid trait. In a mixture of equal volumes of Fletcher trait plasma and patient plasma, about 26% of the normal 1-min arginine esterase activity occurred upon kaolin activation, which was consistent with the prekallikrein content of 28% of normal in the patient plasma.

Confirmatory diagnosis was made by

Table III. Functional clotting of coagulation factors

Factor	Case 1 <sup>1</sup>	Case 2 <sup>1</sup>	Case 3
I mg/dl	330.0	292.0	ND <sup>2</sup>
II	90	ND	80
V %	130	ND	94
VII-X complex,	110	ND	ND
VIII, %	70	ND	ND
IX, %	70	ND	ND
XI, %	88	ND	ND
human deficient plasma	28	28	26
bovine deficient plasma	80	76	ND
XII, %	normal	normal	normal
XIII	28	25	23
Fletcher factor	<1	<1	<1
Fitzgerald factor			

<sup>1</sup> Identical twins.<sup>2</sup> ND = Not determined.Table IV. Effect of kininogen (KGN) preparations upon APTT of patient plasma (PT PL) (case 1)<sup>1</sup>

	APTT sec
Normal plasma + buffer (2 I)	67
PT PL + buffer (2 I)	427
PT PL + bovine HMW KGN (1 µg/ml) (2 I)	74
PT PL + bovine LMW KGN (1 µg/ml) (2 I)	435

<sup>1</sup> After 0.1 ml plasma was incubated with 0.05 ml kaolin (5 mg/ml), 0.05 ml cephalin and 0.05 ml HMW KGN or LMW KGN for 2 min, APTT was recorded by addition of 0.05 ml CaCl<sub>2</sub> (1/40 M).

deficient plasma was used as a substrate (table III)

The clotting defect in this patient was corrected by the addition of highly purified bovine HMW kininogen, but not by the addition of LMW kininogen (table IV).

The kaolin-activated euglobulin lysis time was abnormally prolonged. The addi-

Table V. Kaolin-activated euglobulin lysis time of patient plasma (PT PL) (case 1) and its correction upon mixing with other deficient plasmas

Plasma source	Euglobulin lysis time, min
Buffer control	> 120.0
Normal control	11.0
PT PL	> 120.0
PT PL + normal plasma (1 I)	14.0
PT PL + Hageman trait (1 I)	16.0
PT PL + Fletcher trait (1 I)	16.5
PT PL + Fitzgerald trait (1 I)	> 120.0

tion of Fitzgerald trait-plasma had no effect, whereas the addition of Fletcher trait-plasma corrected the lysis time (table V).

The kaolin-activated esterolytic activity tested on TAME, was extremely low within the first 15 min of exposure to kaolin. With the addition of an equal volume of Fletcher trait plasma to the patient plasma, 1 min arginine esterase activity increased, but only to levels 26% of normal (fig. 2)

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cross-correction studies with Fitzgerald trait-plasma and by the correction of the abnormal APTT by the addition of purified bovine HMW kininogen to patient plasma.

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Table I. Plasminogen levels (proteolytic units/ml) in serum and plasma in normal and eosinophilic subjects

Subjects	Number	Plasminogen, proteolytic units/ml	
		plasma	serum
Normal	98	1.78 $\pm$ 0.38	2.12 $\pm$ 0.35
Male	67	1.78 $\pm$ 0.36	2.10 $\pm$ 0.36
Female	31	1.79 $\pm$ 0.43	2.15 $\pm$ 0.31
Eosinophilia	50	1.79 $\pm$ 0.38	2.14 $\pm$ 0.44
Male	29	1.78 $\pm$ 0.39	2.16 $\pm$ 0.45
Female	21	1.80 $\pm$ 0.35	2.12 $\pm$ 0.44
± Granulocytosis	10	1.82 $\pm$ 0.52	2.20 $\pm$ 0.39
± Idiopathic	14	1.87 $\pm$ 0.26	2.10 $\pm$ 0.42
± Diseases	26	1.73 $\pm$ 0.39	2.14 $\pm$ 0.48

21 females and 29 males, their age range was 11-58 years.

The control group consisted of 98 volunteers, 31 females and 67 males, their age range was 14-66 years. The absolute eosinophil counts were  $0-0.415 \times 10^9/L$ .

#### Plasminogen Determination

Blood was withdrawn after light breakfast. The method previously described by *Olrelami et al.* [5] was partly modified as follows: 0.5 ml of plasma (2 ml of 3.8% sodium citrate and 8 ml of whole blood) or serum were mixed with 0.5 ml of 1/6 N HCl to destroy antiplasmins. The mixture was neutralized by 0.5 ml of 1/6 N NaOH and then activated with 1,000 U of streptokinase (Lederle Laboratories Division, Pearl River NY) at 25-30 °C. The proteolytic activity of formed plasmin was measured by immediately adding 2 ml of 4% casein substrate. After 60 min incubation, the plasminogen level was obtained by the OD difference read at 280 nm in Beckman DU spectrophotometer, converting the OD reading into micrograms of tyrosine and expressed in the proteolytic units of *Remmert and Cohen* [6]. In 5 subjects with eosinophilia and 2 normals, the plasminogen levels were also measured in the buffy coat layer of citrated blood after twice freezing and thawing in dry ice.

#### Bone Marrow Studies

Bone marrow aspirations were performed in 9 eosinophilic patients who had underlying diseases and marked increase in eosinophils in their bone marrow cells was observed.

#### Results and Discussion

As illustrated in table I, there is no significant difference in the plasminogen levels measured either in plasma ( $p > 0.8$ ) or serum ( $p > 0.7$ ) between normals and eosinophils. There are also no significant differences in the plasminogen levels between males and females  $p > 0.8$  for plasma and  $p > 0.4$  for serum, respectively. However there are significantly higher plasminogen levels measured in serum than plasma in both normals and eosinophils with  $p < 0.001$ . The mean plasminogen measured in buffy coat plasma after freezing and thawing was  $1.73 \pm 0.32$  U/ml and no difference was found from the values simultaneously measured in their own plasma with a mean level of  $1.75 \pm 0.37$  U/ml. Plasminogen was stable after freezing [7].

It is known that eosinophilic series are normally the minor component of marrow elements [8]. The increased peripheral blood eosinophils were found along with an increase in the eosinophilic series in the bone marrow [9] as was also observed in this study. If marrow eosinophils are responsible for blood plasminogen synthesis, the increase in marrow eosinophils should



# Plasminogen Levels in Subjects with Eosinophilia

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**Key Words.** Buffy coat Eosinophilia Plasma Plasminogen

**Abstract.** Plasminogen determinations were performed in 98 normal volunteers and 50 subjects with eosinophilia. There was no significant difference in the plasminogen levels between these two groups. Since increased eosinophils in the bone marrow of those eosinophilic subjects were also demonstrated. This observation should raise a question as to whether the eosinophils would really be the site of systemic plasminogen synthesis.

## Introduction

Plasminogen is a plasma protein precursor of the fibrinolytic enzyme plasmin, its function is to lyse fibrin clots. Two major forms of human plasminogen have been identified, one is  $\text{NH}_2$ -terminal glutamic acid with a molecular weight of 87 000 and the other is  $\text{NH}_2$  terminal lysine or valine [1]. In spite of this knowledge, very little has been achieved on the sites of organ synthesis of plasminogen. Liver and kidney apparently did not synthesize plasminogen because the perfusate from the isolated liver perfusion showed no plasminogen activity [2] and normal plasminogen levels were found both in anephric rats and anephric patients [3]. Barnhart and Riddle [4] by immunofluorescent study were able to demonstrate profibrinolysin or plasminogen in the marrow eosinophils, and none in the peripheral blood eosinophils. The marrow eosinophils, therefore, were suggested to be

the site of plasminogen synthesis and before these cells leave for the blood stream they release most of their plasminogen content into the blood. Marrow eosinophils as being a site of plasminogen synthesis has been generally accepted, and no more information is being emphasized to this previous observation. Our present study therefore, will look for the relationship between eosinophil and plasminogen levels by measuring their activity in both normals and subjects with eosinophilia due to various causes.

## Material and Methods

### Subjects

There were 50 subjects with eosinophilia, their absolute eosinophil counts were  $1.153-41.069 \times 10^9/L$ . Of these 50 cases, 10 were associated with clinical gnathostomiasis, 14 were apparently healthy (idiopathic eosinophilia) and 26 were with the following conditions: sepsis, thyrotoxicosis, asthma, accident and cancer. There were

# Erythrocyte and Plasma Lipids in Terminal Renal Insufficiency

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**Key Words.** Erythrocyte lipids Plasma lipids Phospholipid fatty acids  
Terminal renal insufficiency

**Abstract.** Lipids in erythrocytes and plasma of children and adults with terminal renal insufficiency were determined and compared with those of normal controls. The erythrocyte phospholipids in uremic patients were altered in phosphatidylethanolamine (absolutely and relatively elevated) and in lecithin (relatively diminished). Sphingomyelin, phosphatidylserine, total erythrocyte phospholipid content and cholesterol were within the normal range. The detailed analysis of the distribution of plasma phospholipids showed an increase in all phospholipids except phosphatidylserine. The unesterified plasma cholesterol was also found to be elevated. The fatty acid distribution in the individual erythrocyte phospholipids showed the following variations in comparison with controls. one main fatty acid was significantly decreased in sphingomyelin and in lecithin and slightly decreased in phosphatidylethanolamine. It could be shown that the lipid alterations in uremic erythrocytes were quite different from those in plasma. There were no typical signs for an increased lipid peroxidation in the erythrocytes of patients with renal insufficiency

Renal anemia is an early complication of renal insufficiency. It is consistently observed in the presence of serum creatinine levels of 2-3 mg/100 ml [19-22]. A close correlation between glomerulus filtrate and hemoglobin concentration has been noted, in children,  $r = 0.73$  [29]. The two pathogenetic components discussed in renal anemia are an aplastic disorder of the bone marrow related with an erythropoietine deficiency and a shortened erythrocyte survival of

hitherto unknown reason [1, 4, 6, 8, 15, 18-24, 38-40].

In patients with terminal renal insufficiency erythrocyte survival was reduced in 30-60% of the normal value [12, 13]. The hemolytic aspect of anemia in uremia is thought to be caused by alterations in the structure and function of the erythrocytes induced by elevated concentrations of toxic substances in the plasma [1-23, 27, 35, 38-40]. The hemolysis-promoting effect of

theoretically have increased the plasminogen levels. The normal plasminogen levels observed among our eosinophilic subjects in this study should raise a question as to whether marrow eosinophils are really the site of plasminogen synthesis for blood plasminogen Riddle and Barnhart [10] further demonstrated positive plasminogen in peripheral eosinophils from inflammatory tissues and exudate besides marrow eosinophils [2] The positive plasminogen in these particular eosinophils could on the one hand, simply be an inside metabolism in the process of cell maturation and perhaps for their functions. Our present knowledge about eosinophils remains very limited [11] The subjects who exhibited eosinophilia did not necessarily have normally functioning eosinophils and as well as normal plasminogen synthesis. However this finding should lead one to reconsider the site of plasminogen synthesis; especially the eosinophil as being the site of plasminogen synthesis would apparently need further clarification.

Our observation of higher plasminogen activity in serum than in the patients own plasma is interesting. This was different from the observation of Fantl [12] who found the same activity both in plasma and in serum. However he had added the equivalent amounts of sodium citrate that was present in the plasma to the serum before the measurement, while we did not. Since the white blood cells from the buffy coat study appear to have an insignificant plasminogen activity that sodium citrate has an effect on plasminogen measurement has to be kept in consideration.

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( $p < 0.05$ ) increased. Therefore, the proportions of single phospholipids in relation to total phospholipid were altered as follows: decrease in relative phosphatidylcholine (lecithin PCh) content ( $p < 0.01$ ), increase in relative PE content ( $p < 0.01$  tables I, II).

#### Erythrocyte Cholesterol

The cholesterol content of erythrocytes of patients with renal insufficiency was not different from that of controls (table I).

#### Fatty Acid Distribution in Erythrocyte Phospholipids

In comparison with controls, the fatty acid distribution of the three main phospholipids PCh, PE and sphingomyelin (Sph) showed specific variations, whereas that of phosphatidylserine (PS) did not (table III). In uremia, the fatty acid distribution was modified as follows: in PCh, C18:2 was decreased ( $p < 0.05$ ), C24:1/22:4 was slightly increased ( $p < 0.1$ ) in PE, C22:1/20:4 was

Table I. Erythrocyte lipids in uremia. Contents in  $\mu\text{mol P}$  or  $\mu\text{mol cholesterol/ml}$  packed erythrocytes corresponding to packed cell volume

	Uremia			Controls			t	p
	x	s	n	x	s	n		
LL	traces	—	24	traces	—	51	—	
Sph	1.10	0.17	24	1.02	0.18	51	1.29	
PCh	1.19	0.17	22	1.17	0.17	51	n.s.	
PE	1.19	0.18	23	1.06	0.17	51	2.07	$< 0.05$
PS	0.58	0.10	23	0.54	0.11	50	n.s.	
PI	0.03	0.03	24	0.03	0.04	50	n.s.	
Total P	4.07	0.59	23	3.82	0.56	47	1.22	
Chol	3.19	1.07	13	3.26	0.55	21	n.s.	

PI = Phosphatidylinositol total P = total phospholipid content Chol = cholesterol, unesterified x = mean value s = standard deviation n = number of investigations t = value according to Student's t test p = probability of error n.s. = not significant.

Table II. Erythrocyte phospholipids in uremia. Relative contents expressed in percent 100% = total phospholipid

	Uremia			Controls			t	p
	x	s	n	x	s	n		
LL	traces	—	24	traces	—	51		
Sph	26.46	2.09	24	26.66	1.96	52	n.s.	
PCh	28.96	1.43	23	30.78	1.91	52	3.03	$< 0.01$
PE	29.44	1.15	24	27.81	1.95	50	2.90	$< 0.01$
PS	14.41	1.31	24	13.97	1.68	52	n.s.	
PI	2.0	—	24	1.0	—	—		

Abbreviations as in Table I.

the uremic plasma could be investigated by cross-experiments [1 9]

Jacob *et al* [21] Yawata *et al* [38] and Yawata and Jacob [39] found elevated sulfhemoglobin formation in erythrocytes by oxidizing noxes in connection with uremic plasma. The elevated sulfhemoglobin production in uremic erythrocytes could eventually be caused by the influence of substances activated by oxygen [7 34] and disturbances within the pentose-phosphate shunt [21 38-40]. Lipid peroxidation followed by an altered membrane lipid distribution would be the consequence of the two mechanisms described [2, 5 15 16 26 37].

The purpose of this investigation was to examine the lipid distribution in erythrocytes in renal anemia. The major emphasis of the studies outlined in this paper was laid on the problem whether quantitative variations in lipids can explain the reduced erythrocyte survival.

## Material and Methods

The lipids of erythrocytes obtained from 17 children and 7 adults with terminal renal insufficiency were investigated. Plasma phospholipids, plasma free cholesterol and the fatty acid distribution in erythrocyte phospholipids were examined in 15 adults and children with uremia. The results were compared with appropriate controls. 20 ml freshly drawn heparinized blood were analyzed. The investigations were always carried out before hemodialysis. The plasma was separated by centrifugation and the erythrocytes were washed three times in cold isotonic phosphate buffer solution, pH 7.4. Immediately thereafter erythrocytes and plasma were separately homogenized with distilled methanol in an Ultraturrax mixer and after addition of distilled chloroform extracted according to Folch [14]. Butylated hydroxytoluene (Colnbrock Bucks, UK) was added as an antioxidant in each probe and solvent used.

The probes were filtered and evaporated to

dryness under a nitrogen stream and redissolved in chloroform:methanol (2:1, v/v). The lipids were separated by thin-layer chromatography (TLC). Neutral lipids were fractionated by one-dimensional TLC using petroleum ether (180-20, v/v) as solvent; phospholipids were fractionated by two-dimensional TLC, taking the solvent systems chloroform:methanol:ammonia (130:50:10, w/v) and chloroform:acetone:methanol:acetic acid:water (60:80:20:20:10, v/v), respectively.

After visualizing the bands or spots by spraying the plates with an ethanolic solution of dichlorofluorescein (0.2%), the separated probes were scraped off and determined as follows: cholesterol according to Boukers *et al* [3], orthophosphate after digestion of the phospholipids with perchloric acid (2 h, 200 °C) according to Hallmeyer *et al* [17].

The lipid values obtained were related to milliliters of packed erythrocytes corresponding to the packed cell volume (PCV) or to milliliters of plasma.

The fatty acid distribution of the phospholipids was determined according to the classical procedure of Dodge and Phillips [11] with few modifications. The transesterification to methyl esters was performed with BF<sub>3</sub>:methanol (14/1, w/v) according to Morrison and Smith [28].

All analyses were carried out in a Varian, model 2100, gas chromatograph equipped with a hydrogen flame ionization detector and with a Varian integrator. The columns were filled with 10% EGSS-X on Gaschrom-Q 100-120 mesh.

Single fatty acids were identified isothermally at 180 °C; the temperature was programmed between 150 and 190 °C with a heating rate of 1 °C/min for routine estimation. The identification was controlled with NIH test substances. After eliminating values that were out of the normal distribution by the T criterion all the results were interpreted by Student's *t* test.

## Results

### Erythrocyte Phospholipids

Erythrocyte phospholipids of patients with renal insufficiency were slightly but not significantly elevated. Only phosphatidylethanolamine (PE) was significantly

Table V Content of single phospholipids expressed as percent of total plasma phospholipids total plasma phospholipids = 100%

	Uremia			Controls			t
	x	s		x	s		
LL	7.20	2.54	15	6.91	2.36	16	n.s.
Sph	21.94	3.54	15	19.08	2.38	15	n.s.
PCh	64.07	6.10	15	66.44	3.76	15	n.s.
PE	4.41	1.29	15	3.45	1.71	15	n.s.
PS	2.31	1.80	15	3.19	1.33	16	n.s.
PI	-		15			16	

Abbreviations as in table I.

cerides were not determined, because these lipids are not structurally involved in the erythrocyte membrane.

## Discussion

The essential results of our studies involved distinct alterations of erythrocyte phospholipids in patients with terminal renal insufficiency. The PE content was absolutely and relatively increased and in consequence the PCh content was relatively decreased. Moreover PCh, PE and Sph each showed a reduction in one main fatty acid. The cholesterol as the erythrocyte neutral lipid was not modified in patients with uremia.

These results stand out in contrast to the uniform variations in plasma lipids, i.e. a significant increase in plasma phospholipids, with the exception of PS, as well as in unesterified cholesterol. The alterations of erythrocyte lipids noted in uremia thus do not correspond to those of plasma lipids despite the PCh and cholesterol exchange between plasma and erythrocytes [30].

The results further did not show any typical signs of an erythrocyte peroxidation. This can be concluded from the normal or elevated PS and PE contents. The degradation of these lipids by peroxidation is believed to be the consequence of their high amounts of highly unsaturated fatty acids [2, 5, 7, 10, 15, 16, 26, 37]. The fatty acid distribution of PE and PS was only slightly modified in uremia. Only the diminution of arachidonic acid (C20:4) in PE could eventually be associated with peroxidation.

The results of sporadic previous research on erythrocyte phospholipids in renal insufficiency yielded contradictory results. Lutz [25] reported a decrease in total erythrocyte phospholipids in uremia, but his control values were significantly higher (near 50%) than those of our controls and the normal values reported in the literature [10, 11, 30-32]. But he also observed an alteration of the phospholipid distribution in the erythrocytes: the PCh content was relatively decreased, the PE relatively increased.

In contrast to the results of Lutz and to our own results, Plelichowski *et al.* [33] referring their results to the number of eryth-

Table III. Deviated fatty acid amounts of erythrocyte phospholipids in uremia, declared in percent from total fatty acid content (= 100%)

Phospholipid and fatty acid	Fatty acid content						t	p
	uremia			control				
	X	s	n	X	s	n		
Sph C18 2	2.92	1.67	12	1.27	1.23	15	2.06	< 0.05
C22 1/20 4	6.96	3.88	13	3.45	1.36	14	2.22	< 0.05
C24 0	8.12	3.75	14	14.37	3.71	14	2.30	< 0.05
PCh C18 2	15.55	3.23	14	18.61	1.68	13	2.18	< 0.05
C24 1/22 4	2.50	1.71	13	1.26	0.68	13	1.72	< 0.1
PE C22 1/20 4	19.68	3.15	10	22.68	1.43	13	2.07	< 0.1

Abbreviations as in table I.

Table IV. Plasma phospholipids and unesterified cholesterol in uremia. Amounts in  $\mu\text{g}$  P/ml plasma or mg cholesterol/100 ml plasma

	Uremia			Controls			t	p
	X	s	n	X	s	n		
LL	7.37	2.93	15	5.29	1.59	17	1.75	< 0.1
Sph	21.72	4.08	15	13.85	2.81	15	3.70	< 0.001
PCh	66.74	14.59	14	49.89	5.49	15	3.31	< 0.01
PE	4.48	1.74	15	2.71	1.63	15	2.03	< 0.1
PI	2.56	2.37	15	2.49	1.16	16	n.s.	
PI	-	-	15	-	-	15		
Total P	100.47	23.81	15	74.51	8.46	15	2.82	< 0.01
Chol	60.38	11.03	21	50.40	12.28	17	2.60	< 0.05

Abbreviations as in table I

slightly decreased ( $p < 0.1$ ) in Sph, C24 0 was decreased ( $p < 0.05$ ), C18.2 and C22 1/20 4 were increased ( $p < 0.05$ ).

#### Plasma Phospholipids

With the exception of PS the single and total phospholipid contents of plasma were elevated in uremia. lysolecithin (LL) and

PE were slightly elevated ( $p < 0.1$ ) whereas PCh ( $p < 0.01$ ), Sph ( $p < 0.001$ ) and total phospholipid contents ( $p < 0.01$ ) were significantly elevated (table IV). The ratio of single to total phospholipids was normal (table V). Unesterified cholesterol was significantly elevated in uremic plasma ( $p < 0.05$  table IV). Esterified cholesterol and trigly

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rocytes, noted an increase in PCh Sph and total phospholipids, as well as a depletion of PE in erythrocytes of uremic patients. Alterations of erythrocyte membranes involving membrane lipids in uremia could explain the opposite results. The pathogenetic meaning of these changes cannot be interpreted on the basis of our results. Membrane properties as fluidity permeability flux of cations, activity of enzymatic systems are dependent on a normal lipid distribution in the membrane. Finally reference must be made to the fact that the whole erythrocyte population does not obligatorily reflect the alterations of membrane lipids in a few prehemolytic cells, i.e. in part of the population. Further investigations, in particular *in vitro* are designed.

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## Enzyme-Linked Immunosorbent Assay for the Indirect Anti-Human Globulin Test

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**Key Words.** ELISA Antiglobulin test Red cell antibodies

**Abstract.** An enzyme-linked immunosorbent assay has been developed for detecting antibodies combined with red cell surface antigens. In the test, the fluorescence released from 4-methylumbelliferylphosphate is measured after exposure of antibody-coated red cells to alkaline phosphatase coupled to anti-human globulin. The assay is sensitive, reproducible, quantitative and potentially adaptable to an automatic analyzer system.

The agglutinating indirect anti-human globulin test routinely used in hematology and blood transfusion laboratories does not give a quantitative measurement and in some instances is not sufficiently sensitive to detect autoantibodies or alloantibodies capable of destroying red cells of corresponding phenotypes.

The method described here is based on the enzyme linked immunosorbent assay [1] with the antibody-coated red cells as the immobile immunosorbent phase. Alkaline phosphatase covalently coupled to anti-human globulin enables a quantitative measure of antibodies by using the fluorescent substrate 4-methylumbelliferylphosphate. The technique is reliable, quantitative, and about four times more sensitive than the routine method.

### Materials and Methods

Human blood was collected in acid-citrate-dextrose solution and kept for up to 30 days at refrigerator temperature before washing and resuspension as described below. Anti-D and anti-c sera were preserved in 0.1% sodium azide and kept frozen until use. Their titers by the standard manual antiglobulin technique were 1:32 and 1:16, respectively when tested against *CDe/CDe* (for anti-D) and *cde/cde* (for anti-c) red cells. (The statistically most probable genotypes are given here to show single or double representation of specific Rh antigens on the red cells. The likelihood of Rh genotyping error of *CDe/CDe* cells is about 2%, since there is no anti-d antibody.)

#### *Rabbit Anti-Human Globulin*

Plasma from 4 donors was pooled and the immunoglobulin fraction purified by ammonium sulphate precipitation and extensive dialysis [2]. Rabbit antiserum to this human immunoglobulin was

prepared and the rabbit immunoglobulins purified as previously described [3]. This rabbit immunoglobulin was then coupled to alkaline phosphatase (EC 3.1.3.1, calf intestine, 35 U/mg, Boehringer) using glutaraldehyde [4]. In typical experiment 10 mg alkaline phosphatase was coupled to 5 mg immunoglobulin by 0.15% w/v glutaraldehyde in 0.1 M Na phosphate, pH 7.0, to final volume of 2 ml for 120 min at 23 °C. The reaction was terminated by the addition of 2 ml of 0.2 M ethanolamine pH 7.0, and then dialyzed against 1 liter of this solution, followed by two dialyses against 0.1 M NaCl. The coupled antibody-alkaline phosphatase complex was made to final volume of 20 ml with 0.1 M NaCl/0.1% NaN<sub>3</sub>. This solution was stable for at least 6 months.

#### Low Ionic Strength Solution (LISS) [5]

A concentrated stock solution was made of 0.3 M NaCl, 2.4 M glycine, 30 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.05% NaN<sub>3</sub> titrated to pH 6.2 with NaOH. When diluted tenfold this gave working solution at pH 6.7.

#### 4-Methylumbelliferylphosphate Solution

This stock substrate buffer was made by titrating 0.15 M NaHCO<sub>3</sub> with 0.15 M NaOH to pH of 9.8. To this buffer was added MgCl<sub>2</sub> to concentration of 5 mM and 4-methylumbelliferylphosphate (Sigma) at 1 mM to give the final substrate solution. This solution was stable for at least 10 h at room temperature.

#### Wash Solutions

These were 1% BSA (bovine serum albumin; Sigma, fraction V) in normal saline (0.9% w/v) plus 0.05% NaN<sub>3</sub>, or 1% BSA in LISS.

#### Silicon-Coated Test Tubes

Pyrax tubes 10 × 100 mm were coated with silicon by soaking in 5% solution of Pierce Dv-Film in toluene, and then air-dried.

#### Fluorescence Assay

Red blood cells were washed four times with saline (0.9% w/v) and once with LISS. The cells were sensitized by incubating one drop of the centrifuged cells in the silicon-coated test tubes with 1 ml of LISS-diluted antiserum for 20 min at 37 °C. For control, washed unsensitized cells

were incubated in either LISS, 1% BSA in LISS, or normal serum diluted in LISS. After incubation, the cells were washed five times in 1% BSA/LISS, with vigorous injection of wash solution into the test tube, followed by centrifugation and aspiration. The control and sensitized cells were then incubated with 40 µl anti-human globulin-alkaline phosphatase conjugate in 0.15 ml 1% BSA/LISS for 40 min at 37 °C, followed by four washings with 1% BSA/saline and two with saline alone. To measure the complexed alkaline phosphatase, 0.7 ml of 1 mM 4-methylumbelliferylphosphate solution was added. After re-suspension of the red cells, the reaction proceeded at room temperature for 50 min with the tubes rotating at about 1 rev/sec at an angle of 45° to the horizontal. The tubes were then centrifuged and 0.3 ml of the supernate was added to 1.0 ml of 0.15 M NaHCO<sub>3</sub>/NaOH solution, pH 10.3. The fluorescence was recorded with Turner model 430 spectrofluorimeter at 365 nm excitation and 453 nm emission [6]. The results were expressed as fluorescence units representing the reading of the sensitized cells minus that of the controls.

## Results and Discussion

Figure 1 shows the results of experiments performed on separate occasions with the red cells of 3 different CDe/CDs donors and serial dilutions of the stock anti-D serum. The data points are the mean of four determinations, and the fluorescence plotted is that of the sensitized minus the control cells. The fluorescence of the control cells did not vary with the composition of the first incubation solution, being the same in LISS, 1% BSA/LISS or anti-c serum diluted in LISS. The coefficient of variation for these determinations was about 5%, and the variances of the controls and the three most dilute antisera were essentially the same. The variances of the control samples were combined to give a standard deviation of  $S = 2.8$  fluorescence units ( $n = 12$ )

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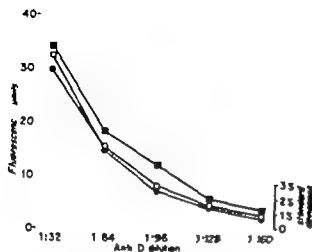


Fig. 1. Enzyme linked assay of the red cells of 3 *CDe/CDe* donors. The cells had been stored for 3 days (■), 12 days (○) and 30 days (●). The unit of standard deviation was derived from the pooled variance of the controls.  $n = 12$ .

Table I. Mean of duplicate determinations of fluorescence, using red cells homozygous and heterozygotes for the *c* antigen

Anti-c dilution	Red cell Rh type		Ratio <i>cde/cde CDe/cde</i>
	<i>cde/cde</i>	<i>CDe/cde</i>	
1 16	16.3	7.2	2.3
1 32	6.2	3.4	1.8

which was used to construct the right-hand ordinate in figure 1. At an anti-D dilution of 1:96 the experiment with the lowest response had a fluorescence about 2.3 standard deviations greater than that of the control; thus the probability of a false reading was less than 1%. At a dilution level of 1:64 the probability of false evaluation was negligible. Using the manual indirect agglutinating test with each of the three red cell sam-

ples, the titer was 1:32. Red cell age of up to 30 days did not appreciably alter the magnitude of the response in the assay. However, older cells were more fragile and could hemolyze, with resultant quenching of the fluorescence by the released hemoglobin. To reduce this quenching effect the reaction mixture was diluted in buffer to pH 10.3, the pH of maximum fluorescence, before measurement.

Red cells of genotypes *CDe/cde* and *cde/cde* were sensitized with anti-*c* to measure the dosage effect in homozygotes and heterozygotes. The results given in table I are expressed in fluorescence units as the mean of duplicate experiments. They show a doubling effect of cells with single and double dosage of the *c* antigen. When these red cells were treated with anti-*c* and the usual indirect antiglobulin test, the *CDe/cde* cells were only very weakly agglutinated and the titer of the serum against *cde/cde* cells was 1:16.

In this immunosorbent method the variables with greatest effect are the amount of BSA in the second incubation solution, and the volume of coupled antibody-alkaline phosphatase solution. In the absence of BSA the nonspecific adsorption of the antibody-alkaline phosphatase complex to red cells is extremely high, resulting in no difference between sensitized and control cells. Increasing the volume of enzyme-linked antibody can decrease the second incubation time and increase the sensitivity. The volume chosen was a compromise between reagent use and reaction time. This assay can be completed in about 2 h and leads to a quantitative and sensitive indirect anti-human globulin test that also has the potential for use in an automatic analyzer system.

clinically significant complement activation does not occur during this procedure — a fact of some importance because of concerns for donor safety. The pattern of leukocyte counts during IFCL has not been reported. Accordingly we performed serial total and differential white blood cell counts (WBC) during this procedure.

### Methods

This study was approved by the local committee concerned with human investigations, and informed consent was obtained. IFCL was performed as described [7] using the Haemonetics Model-30 Blood Processor (Haemonetics Corp. Natick, Mass.) employing 6% hydroxyethyl starch (Voltec, McGraw Laboratories, Irvine, Calif.) rendered 7% with respect to treosulfon citrate. Total and differential WBC were performed before and after IFCL in 25 consecutive donors. During 8 IFCL procedures seven samples of donor venous blood were collected serially: one just before beginning (PRE), and six at timed intervals during the procedure (numbered 1-6). Number 1 was taken at the start of the second collection cycle as follows. The cell-free plasma and erythrocytes obtained from the first cycle were reinfused into the donor over 7-min interval, and the sample was obtained seconds later. Theoretically this reinfused material should contain any complement activated via contact of donor blood with the machine. Samples numbered 2 and 3 were taken 5 and 10 min later. Thus, samples were obtained 7, 12, and 17 min after beginning the reinfusion of IFCL processed blood, and it is likely that complement-induced neutropenia would be detected by samples timed in this fashion since this phenomenon occurs 10-20 min after beginning reinfusion of CFFL processed blood [1, 4, 5]. Cells were collected by standard procedure during the third cycle, and WBC were not performed. Samples 4, 5 and 6 were obtained during cycle four in fashion analogous so that in the second cycle (number 4 at the start, 5 and 6 after 5 and 10 min of collection) to monitor WBC after second reinfusion of IFCL processed blood.

Leukocytes were counted electronically total WBC using the Hemalog 8 and differential WBC with the Hemalog D (Techicon Incorporated, Terrytown, N.Y.). Data were evaluated by paired analysis employing the sign and paired *t* tests.

### Results

25 donors underwent complete IFCL (8 collection cycles). Total WBC and neutrophil counts in donor blood were significantly decreased ( $p < 0.05$ ) after the procedure, although changes expressed as mean  $\pm$  SEM were, in fact, slight. Prepheresis total WBC and neutrophil counts were  $5,288 \pm 281$  and  $2,994 \pm 197$  cells/ $\mu$ l, whereas, postpheresis values were  $4,984 \pm 227$  and  $2,919 \pm 177$  cells/ $\mu$ l, respectively.

Changes in leukocyte counts in the blood of 8 donors as measured serially during IFCL are presented in table I. Every leukocyte type decreased significantly (sign and/or paired *t* test) in concentration following the reinfusion of cell-free plasma and erythrocytes obtained from the first collection cycle. Although the decreases were small for total WBC, neutrophil and lymphocytes, the concentrations of these cells remained below prepheresis values, almost without exception, throughout the procedure (table I). Monocytes, eosinophils and basophils also fell below prepheresis values, but they did not remain consistently lower than prepheresis ones. However a high degree of counting precision was impossible, as evidenced by the relatively large standard errors, because cell concentrations were low.

The concentration of each type of leukocyte present in one sample was compared to the corresponding value in the next (e.g., neutrophils in 1-vs-neutrophils in 2, -vs. 3 etc.). With the exception of the significant



## Effect of Intermittent-Flow Centrifugation Leukapheresis on Donor Leukocyte Counts

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**Key Words.** Complement. Intermittent flow centrifugation leukapheresis. Leukapheresis. Leukocyte counts.

**Abstract.** Effects of intermittent-flow centrifugation leukapheresis (IFCL) on leukocyte counts in donor blood have not been reported. Accordingly total and differential white blood cell counts were performed serially during IFCL. A significant decrease ( $p < 0.05$ ) in all types of leukocytes occurred in donor blood early in pheresis and was probably the consequence of hemodilution. Concentrations of most leukocyte types remained decreased throughout the procedure, and a rebound leukocytosis was not detected. Thus, leukocyte kinetics in donor blood during IFCL differ from those reported for filtration leukapheresis in which the pattern of selective neutropenia followed by rebound neutrophilia has been ascribed to complement activation.

### Introduction

Neutropenia followed within minutes by return towards, and often above, normal values, occurs in donor blood during the early moments of continuous-flow filtration leukapheresis (CFFL) [4-6]. This reaction has been explained as follows: (a) complement is activated by the interaction of blood with nylon filters; (b) cleavage peptides (C5a) are infused into the donor via the blood return line and promote the formation of neutrophil aggregates that are transiently sequestered within the microvasculature, particularly pulmonary vessels [1, 2, 4]. This

pattern of selective neutropenia followed by rebound neutrophilia has been employed as evidence to support complement activation during hemodialysis and in animal models [reviewed in 2].

Complement changes are seen during intermittent flow centrifugation leukapheresis (IFCL) however they have been ascribed, not to activation, but to the loss of proteins by adsorption onto the artificial surfaces of the IFCL apparatus [8]. Subtle evidence of complement activation could pass undetected by *in vitro* studies, and the absence of changes in donor neutrophil counts during IFCL would provide further evidence that

Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
$4.3 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$4.3 \pm 0.2$ (NS) ( $p < 0.05$ )	$4.2 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$4.3 \pm 0.3$ (NS) (NS)	$4.2 \pm 0.2$ ( $p < 0.05$ ) ( $p < 0.01$ )
$2.4 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$2.4 \pm 0.1$ ( $p < 0.05$ ) ( $p < 0.05$ )	$2.4 \pm 0.1$ (NS) ( $p < 0.01$ )	$2.5 \pm 0.2$ (NS) (NS)	$2.4 \pm 0.1$ ( $p < 0.05$ ) (NS)
$1.3 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$1.3 \pm 0.1$ (NS) (NS)	$1.3 \pm 0.1$ ( $p < 0.05$ ) ( $p < 0.05$ )	$1.2 \pm 0.1$ ( $p < 0.05$ ) ( $p < 0.05$ )	$1.3 \pm 0.1$ (NS) (NS)
$337 \pm 16$ (NS) ( $p < 0.05$ )	$341 \pm 23$ (NS) (NS)	$325 \pm 30$ (NS) (NS)	$343 \pm 36$ (NS) (NS)	$356 \pm 28$ (NS) (NS)
$124 \pm 7$ (NS) ( $p < 0.05$ )	$118 \pm 8$ ( $p < 0.05$ ) ( $p < 0.05$ )	$123 \pm 4$ ( $p < 0.01$ ) ( $p < 0.01$ )	$121 \pm 5$ ( $p < 0.05$ ) ( $p < 0.01$ )	$125 \pm 7$ (NS) (NS)
$35 \pm 2$ ( $p < 0.01$ ) ( $p < 0.01$ )	$39 \pm 4$ (NS) (NS)	$34 \pm 3$ ( $p < 0.01$ ) ( $p < 0.01$ )	$39 \pm 4$ (NS) (NS)	$38 \pm 6$ (NS) (NS)

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decrease noted when sample 1 values were compared to prepheresis counts, no significant changes in any leukocyte type occurred later during the procedure ( $p > 0.05$ ) indicating that neither a rebound leukocytosis nor an additional fall could be detected in sequential samples

## Discussion

A significant decrease in all types of leukocytes occurred in donor blood early in IFCL. Concentration of several leukocyte types remained low throughout the procedure. Rebound leukocytosis was not detected. This slight leukopenia, involving all types of leukocytes, is probably the result of volume expansion since 10–20% hemodilution has been repeatedly documented during IFCL. By the technique employed in this study [7] donor hematocrits consistently fall 10–12% [3–9]. Unlike the situation in CFFL, complement activation seems unlikely in IFCL because of the lack of a selective and marked (< 50% decrease) neutropenia followed by neutrophilia [4–6]. These data support previous information that complement is not activated during IFCL [8] and suggest that, at least in this respect, IFCL poses less donor risk than CFFL.

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Table I. Leukocyte changes in sequential samples during intermittent-flow centrifugation leukapheresis

Leukocyte	Prepheresis	Sample 1
Total WBC	$4.8 \pm 0.5$	$4.1 \pm 0.1^1$ ( $p < 0.01$ ) ( $p < 0.01$ )
Neutrophils	$2.6 \pm 0.4$	$2.1 \pm 0.2$ ( $p < 0.05$ ) ( $p < 0.05$ )
Lymphocytes	$1.5 \pm 0.2$	$1.3 \pm 0.2$ ( $p < 0.05$ ) (NS)
Monocytes	$381 \pm 43$	$281 \pm 36^2$ ( $p < 0.01$ ) ( $p < 0.05$ )
Eosinophils	$141 \pm 33$	$90 \pm 23^2$ ( $p < 0.01$ ) (NS)
Basophils	$45 \pm 7$	$44 \pm 15^2$ ( $p < 0.05$ ) (NS)

<sup>1</sup> Cells  $\times 10^9/\mu\text{l} \pm \text{SE}$  (significance of prepheresis vs. sample by sign test) (significance of prepheresis vs. sample by paired *t* test).

<sup>2</sup> Total cells/ $\mu\text{l}$  rather than cells  $\times 10^9/\mu\text{l}$ .

Institutes of Health. Dr. Maguire is a Clinical Fellow of the American Cancer Society

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Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
$4.3 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$4.3 \pm 0.2$ (NS) ( $p < 0.05$ )	$4.2 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$4.3 \pm 0.3$ (NS) (NS)	$4.2 \pm 0.2$ ( $p < 0.05$ ) ( $p < 0.01$ )
$2.4 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$2.4 \pm 0.1$ ( $p < 0.05$ ) ( $p < 0.05$ )	$2.4 \pm 0.1$ (NS) ( $p < 0.01$ )	$2.5 \pm 0.2$ (NS) (NS)	$2.4 \pm 0.1$ ( $p < 0.05$ ) (NS)
$1.3 \pm 0.1$ ( $p < 0.01$ ) ( $p < 0.01$ )	$1.3 \pm 0.1$ (NS) (NS)	$1.3 \pm 0.1$ ( $p < 0.05$ ) ( $p < 0.05$ )	$1.2 \pm 0.1$ ( $p < 0.05$ ) ( $p < 0.05$ )	$1.3 \pm 0.1$ (NS) (NS)
$337 \pm 16$ (NS) ( $p < 0.05$ )	$341 \pm 23$ (NS) (NS)	$325 \pm 30$ (NS) (NS)	$343 \pm 36$ (NS) (NS)	$356 \pm 28$ (NS) (NS)
$124 \pm 7$ (NS) ( $p < 0.05$ )	$118 \pm 8$ ( $p < 0.05$ ) ( $p < 0.05$ )	$123 \pm 4$ ( $p < 0.01$ ) ( $p < 0.01$ )	$121 \pm 5$ ( $p < 0.05$ ) ( $p < 0.01$ )	$125 \pm 7$ (NS) (NS)
$35 \pm 2$ ( $p < 0.01$ ) ( $p < 0.01$ )	$39 \pm 4$ (NS) (NS)	$34 \pm 3$ ( $p < 0.01$ ) ( $p < 0.01$ )	$39 \pm 4$ (NS) (NS)	$38 \pm 6$ (NS) (NS)

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## *L*- $\gamma$ -Glutamyl Transpeptidase Activity in Normal and Leukemic Leukocytes

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**Key Words.**  $\gamma$ -Glutamyl transpeptidase Leukemia Leukocytes

**Abstract.** The activity of  $\gamma$ -glutamyl transpeptidase was investigated in normal and leukemic leukocytes. Enzyme activity was positively correlated with the proportion of mature neutrophils and monocytes. In isolated leukocytes from patients with acute myeloid leukemia low values were obtained compared to controls.

### Introduction

Simple, synthetic, commercially available substrates have been shown to be valuable in the study of many different enzymes in human white blood cells [1 2]. Thus, analysis of the leukocyte content of *N*-acetyl  $\beta$ -glucosaminidase seems to facilitate the morphologic diagnosis of the monocytic leukemias even when they present with very immature precursor cells in the peripheral blood.

The present investigation was undertaken to study the distribution of a membrane enzyme, i.e.,  $\gamma$ -glutamyl transpeptidase in normal and leukemic leukocytes.

### Nomenclature

The classification of the leukemias and of the different cell types is that of Sjögren [5]

### Material and Methods

**Controls.** These comprised 4 men and 4 women (laboratory personnel) aged 21-57 years.

**Patients.** All the patients were investigated at the time of diagnosis. The diagnoses and peripheral blood data are presented in table I.

**Morphological Investigations.** May-Grünwald-Giemsa stained blood films from all patients and concomitant bone marrow smears from 18 patients were available. A differential count of 1,000 nucleated cells in each smear was performed by one of the authors (U.S.) without knowledge of the biochemical data.

**Enzyme Assay**  $\gamma$ -Glutamyl transpeptidase was determined by the method of Baker Chemicals BU (Deventer Holland) with *L*- $\gamma$ -glutamyl *p*-nitroanilide as substrate and using 100  $\mu$ l of leukocyte homogenate (5,000-10,000 leukocytes/ $\mu$ l prepared as described earlier [1]) as enzyme source.

**Statistics.** Nonparametric statistics were employed. The Mann-Whitney two-tailed U test and the Spearman two-tailed rank correlation test were used to assess significance of the results.

Table I. Hematologic data of 23 patients and of 8 normals

	Sex	Age	Diagnosis	γ-Glutamyl trans- peptidase <sup>1</sup>	WBC	10 <sup>9</sup> /l	N %	E %	L %	M %	PM %	MB %	MC %	LB %
<b>Patients</b>														
H.N.	M	66	reactive eosinophilia	4.73	38.5		29.2	61.8	5.5	3.0				
Y.L.	M	28	reactive eosinophilia	7.56	9.4		33.8	15.3	44.8	4.5	1.1			
A.H.	M	62	B cell lymphoma	4.56	5.4		39.4	1.2	22.0	4.8			27.6	5.0
H.I.	M	III	AML MO	0.88	240.0		3.4	0.1	1.9	0.7	1.0	90.6	1.5	0.7
H.L.	F	52	AML MO	0.78	161.0		5		3.0		0.2	92.9	1.4	
A.H.	F	63	AML M1	6.19	227.0		12.6	0.4	34.0	1.4	9.4	36.1	5.9	
R.S.	M	38	AML M1	7.14	26		7.6	0.1	5.6	0.2	4.4	79.4	2.7	
B.H.	M	4	AMMoL M4M	1.51	16.5		2.8		10.0	14.0	39.0	16.2	17.4	1.2
O.H.	M	70	AMMoL M4	2.10	24.3		35	1.5	1.5	3.5	22.4	6.4	14.2	
M.K.	F	III	AMMoL M4Eo	8.09	64.0		9.7	3.1	30.3	24.3	15.9	16.0	0.7	
W.W.	M	74	CMMoL	14.4	14.1		35.7	1.5	28.1	29.1	4.9		0.2	
E.R.	M	60	smouldering leukemia	7.39	11.9		37.1	0.4	16.2	3.2	7.7	29.4	3.4	0.4
S.W.	F	74	AMoL M5B	9.66	7.5		21.8		19.8	22.6	34.0	1.6	0.2	
N.E.A.	M	48	AMoL M5B	3.99	8.4		3.7	1.8	33.2	5	22.1	31.4	5.3	
H.C.	M	77	AMoL M5B	3.78	152.0		1.2	0.1	4.7	0.4	53.8	37.6	2	
K.R.	F	75	AM L M5B	1.03	64.5		5		6.9	0	11	74.1	2.0	
U.P.	M	43	ALL	15.8	1.9		3.8	0.5	64.5	3.2	0.8		0.5	26.5
L.P.	F	26	ALL	73	18.5		2.6		33.7	0.3	0.5		1.1	61.8
P.S.	M	22	ALL	0.10	43.0		19.4	2.0	13.8	2.8	0.8	0.6	2.0	58.6
T.S.	M	57	CLL	9.24	43.3		4.4	0	94.6	0.8				
O.B.	F	82	CLL	8.08	23.4		18	4.4	74.6	2.8				
E.B.	F	71	CLL	23.1	133.0		3.1	0.1	93.8	1.0				
L.L.	M	76	CLL	0.022	90.0		3.7	0	95.3	0.2			0.2	0.4
<b>Normals</b>														
(range) 4M/ 21 57				5.00-16.8	5.6-9.9		48	0.5	21.4	3.4				
4F							-68.3	7.7-45.8	-8.3					

N = Neutrophils E = eosinophils L = lymphocytes M = monocytes PM = promonocytes MB = myeloblasts MC = promyelocytes-metamyelocytes LB = lymphoblasts AML = acute myeloblastic leukemia AMMoL = acute myelomonocytic leukemia CMMoL = chronic myelomonocytic leukemia AMoL = acute monocytic leukemia ALL = acute lymphoblastic leukemia CLL = chronic lymphocytic leukemia. The subtypes according to the FAB classification system [5] are also given.

γ-Glutamyl transpeptidase activity is expressed as micromoles substrate split per minute per liter leukocyte homogenate.

## Results

Enzyme value, diagnosis, and peripheral blood data are shown in table I. The relations between  $\gamma$ -glutamyl transpeptidase activities and the proportions of different types of leukocytes are presented in table II. It can be seen that enzyme activity was positively correlated with the proportions of mature neutrophils and monocytes.

The enzymic activities of the acute myeloid leukemias patients were significantly lower than those of the normal controls ( $p < 0.01$ ). Within the lymphatic disease group there was great variability in enzyme activity.

Table II. Relations between enzymic activities expressed as U/10<sup>9</sup> white blood cells and percentages of different types of white blood cells. Figures give the Spearman rank correlation coefficients and the degrees of significance.

Cell types	All patients and controls (n = 31)	All non-lymphatic diseases (n = 23)
N	0.635 < 0.001	0.798 < 0.001
N + M	0.685 < 0.001	0.861 0.001
N + E	0.595 < 0.01	0.708 < 0.001
N + MC	0.521 < 0.01	0.744 < 0.001
N + M + MC + PMo	0.454 < 0.02	0.545 < 0.01

N = Neutrophils M = monocytes E = eosinophils MC = promyelocytes-metamyelocytes PMo = promonocytes.

Table III. Relation between alkaline phosphatase and L- $\gamma$ -glutamyl transpeptidase.

	R <sub>s</sub>	Significance
All patients and controls (n = 31)	0.337	n.s.
All nonlymphatic diseases (n = 23)	0.579	p < 0.01

Alkaline phosphatase was also analyzed in all patients and controls and the results were shown in a previous study [2]. There was no significant correlation between  $\gamma$ -glutamyl transpeptidase and alkaline phosphatase when all patients and controls were included in the test (table III). When the lymphatic diseases were excluded, a significant correlation was obtained (table III).

## Discussion

$\gamma$ -Glutamyl transpeptidase is a cell membrane enzyme with high activity in epithelial cells of many organs, e.g., the liver and the kidneys. This enzyme is also present on the external surface of lymphoid cells with a marked range of activity depending on the subpopulations defined by membrane phenotype [3-4]. In general, cell lines from patients with lymphoproliferative diseases had lower activities than corresponding lines from controls [3-4].

$\gamma$ -Glutamyl transpeptidase has formerly been histochemically demonstrated in myeloid elements of the bone marrow including mitotic normoblasts and megakaryocytes [7]. The  $\gamma$ -glutamyl transpeptidase activity of the marrow lymphocytes was also shown to be significantly lower than that of the lymph node lymphocytes.

Using subcellular fractionation of isolated granulocytes, Rustin and Peters [6] have recently shown that γ-glutamyl transpeptidase exhibits a binodal distribution with localization to both endoplasmic reticulum and specific granular components. Alkaline phosphatase activity showed a similar distribution.

In the present investigation we found a pronounced activity in the mature neutrophils and monocytes. Lower values were recorded in the eosinophils and the granulopoietic precursor cells. Low enzymic activity has formerly been recorded in the acute myeloid leukemias [3] and this is verified in the present paper. In the acute lymphoid leukemias even raised enzymic activities may occur probably caused by differences in the cell-surface structure and its immunological characteristics.

Alkaline phosphatase was also analyzed in all patients and controls [2]. This activity was most pronounced in the mature neutrophils. When the lymphatic diseases were excluded, there was a significant correlation between the two enzymes.

The surface localization of γ-glutamyl transpeptidase [3-4] makes this enzyme useful as a surface marker for normal and neoplastic cells in various stages of differentiation and maturation. A combination of analyses of γ-glutamyl transpeptidase with some hydrolases, e.g. the phosphatases and the N-acetyl β-glucosaminidase makes it possible to obtain enzyme profiles with characteristic patterns for the various types of myeloid and lymphoid leukemias. Studies of the enzyme content may facilitate the morphological classification besides the use of various cytochemical staining procedures necessary for the separation of the acute leukemias into several cytological subgroups with different therapeutic regimes.

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## Plasma cell Leukaemia

### Diagnostic Problems In Our Experience with 11 Cases

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**Key Words.** Malignant plasma cell dyscrasias · Plasma cell asynchronism · Plasma cell leukaemia · Transmission electron microscopy

**Abstract.** 11 patients with plasma cell leukaemia (PCL) are reported. Diagnostic clinical, haematological, immunological, biochemical and electron microscopical (TEM) data were analysed and compared to the largest series of PCL cases reported in the literature. Special attention was paid to four facets of this disease: (a) the clinical picture at admission, (b) the frequency of PCL, (c) the production of M components in relation to the maturity and type of the asynchronous plasma cells, and (d) the diagnostic problems of this entity of acute leukaemia of the afferent limb of the B lymphocyte transformation. In this series PCL emerges as a distinct clinical entity: patients are severely anaemic, hepatosplenomegaly is prominent, bone lesions are uncommon but if present are usually non-osteolytic, and the response to treatment with an alkylating agent and glucocorticoid is poor. The diagnosis is difficult since the circulating plasma cells may have morphological features which only allows the diagnosis to be made after the TEM examination. If the peripheral blood of cases of acute leukaemias and immunocytic dyscrasias is routinely examined by TEM, PCL appears to be a not uncommon variant of plasma cell dyscrasia - in the present study it was 11%.

### Introduction

The first light microscopic description of plasma cells that conforms with our present day criteria comes from von Marschalko [34] in 1895, although plasma cells were recognized by Ramon y Cajal [21] and Unna [33] in 1890 and 1891, respectively. Plasma cell leukaemia (PCL) is a malig-

nant plasma cell dyscrasia due to the proliferation of these cells in the bone marrow, peripheral blood and visceral organs. It is a distinct clinicopathological condition presenting characteristically with hepatomegaly and/or splenomegaly, relatively little skeletal damage and an acute course with a mean survival of 4.8 months from the time of diagnosis [15]. It is a rare disease, first

described by Foa [7] in 1904 with an incidence 1.6-2% that of multiple myeloma [5 11 14] 111 acceptable cases of PCL have been reported in the literature [5 11 12, 14 15-18, 23 26, 36] Only the very recently published series includes 8 fully investigated patients from the clinical, haematological, immunological, biochemical and electron microscopical (TEM) point of view [36] It is generally acknowledged that TEM can play an important role in identification of plasma cells and thus may facilitate the diagnosis of PCL.

We are reporting 11 cases of PCL fulfilling diagnostic criteria, and will focus on problems pertaining to: (a) the clinical picture at admission (b) the frequency of PCL, (c) the production of M components in relation to the maturity of plasma cells, and (d) the diagnostic problems of this entity of acute leukaemia of the afferent limb of the B lymphocyte transformation.

## Material

### Patients

11 cases of PCL were diagnosed in the local academic hospitals during the period April 1974 to September 1978. Details of relevant clinical and radiological features (table I), haematological features (table II) and immunological and biochemical features (table III) of patients are given. Reported patients in this article were all diagnosed as having PCL on admission into hospital, and none of these patients suffered from any other malignant plasma cell dyscrasia before the diagnosis of PCL.

## Methods

### Definition / PCL

PCL represents separate clinicopathological entity of immunocytic dyscrasies. The diagnosis of this disease is difficult if all cases are to be diag-

nosed. In order to overcome difficulties in problematic cases, electron microscopy must be used, as is clearly seen from the present study. The diagnostic criteria of this disease was formulated and used in the past by others (see 'Introduction') and the same were also applied in this study. We would like to stress that the criterion of the obligatory presence of 20% of plasma cells in the peripheral blood established by the light microscopy should be verified electron microscopically.

### Preparation and Processing for TEM

The preparation and processing of the peripheral blood and bone marrow for TEM was the same as described for the morphological identification of T and B lymphocytes [27 28] and recently used for studies from the field of functional ultrastructure [30, 31].

### Assessment / Synchronous or Asynchronous Plasma cells

Describing the B lymphocyte transformation stages during an immune response we defined the morphological characteristic of normal (reactive) plasma cell (PC) [28].

In their study of 57 patients with various plasma cell malignancies and 25 patients with other diseases Graham and Bernier [10] introduced the diagnostic and prognostic values of asynchronous plasma cells in plasma cell malignancies.

We also studied plasma cells in 30 patients with malignant plasma cell dyscrasies, and 44 patients with other diseases, and we can confirm most of Graham and Bernier findings. One of us (AT) enlarged the concept of asynchronous plasma cells and established the law for the plasma cell maturation [7]. Briefly many morphological variants of asynchronous plasma cells have been identified, but for diagnostic purposes two are essential, namely the asynchronous plasma cell of type I or shortly PCI and asynchronous plasma cell of type II or shortly PCII [27] (Fig. 1).

A plasma cell having more than half of its cytoplasm filled with rough endoplasmic reticulum (ER) and fine, homogeneous chromatin pattern in the nucleus (without clumps of heterochromatin) is called PCL, hence a cell with similar cytoplasmic characteristics but with heterochromatin clumps of small size attached to or free from the inner nuclear membrane is called PCII, PCI and PCII were quantitated in the peripheral blood of

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Table I Clinical data at admission to the hospital and the prognosis of 11 patients with PCL

Patients		Age years	Sex	Race	Hospitalised	Anaemia Haemorrhage	Bone pain	Bone X-ray	Hyper viscosity
No	name								
1	J W B	43	M	caucasoid	28.3.74	moderate yes	no	normal	severe
2	P M	60	M	negroid	18.8.74	moderate no	yes <sup>a</sup>	osteop.	no
3	P S	73	M	negroid	6.1.75	severe yes	no	osteop.	no
4	B M	43	M	negroid	15.3.75	moderate yes	- <sup>b</sup>	normal	no
5	J N	73	M	negroid	4.9.75	moderate yes	no	osteop.	no
6	J N	19	M	negroid	21.9.75	severe yes	no	osteop.	no
7	A F	33	M	negroid	3.10.75	severe yes	- <sup>b</sup>	ND	no
8	E T	36	F	negroid	4.11.75	severe yes	no	normal	no
9	M M	89	F	negroid	28.2.76	severe no	yes	osteoly	no
10	E M	50	F	negroid	8.6.77	severe no	yes	osteoly	no
11	P B.W	67	M	caucasoid	10.1.78	no no	yes	osteoly	no

Only after pressure on the sternum.

<sup>b</sup> Comatous. Not assessed

Table II. Haematological data at admission to the hospital of the 11 patients with PCL

Pa Peripheral blood  
tient  
No. light microscopy (LM)

	leukoc. × 10 <sup>9</sup> /l	Hb g/l	Hct %	MCV fl	MCH pg	MCHC	Thr	Retic 10 / 1	ESR × 10 <sup>3</sup> /l	NG West %	EG %	BG %	L %	M %	BLAST %
1	9.4	7.7	22	86	30	35	108	66	150	38 <sup>a</sup>	2	1	46 <sup>a</sup>	9	
2	66.1	7.3	19	100	37	33	205	48	17	9				91	
3	8.1	3.1	10	91	28	31	48	30	8	59			40	1	
4	3.5	7.5	1	84	29	35	89	125	25	8					90
5	6.5	7.3	23	106	34	32	51	72	155	49 <sup>a</sup>			46	3	
6	87.6	5.0 <sup>a</sup>					23	4	45	3			1		96
7	48.0	4.6	13	86	30	35	29	46	110	4				1	86
8	1.4	4.4	14	82	26	33	4	48	40	33	3	1		3	52
9	5.3	4.2	14	120	38	32	108	12	170	44			49	5	
10	37.0	4.5	14	99	33	34	50	37	100	11					117
11	6.7	12.6	36	91	32	35	250	ND	11	50	2		12	9	77

a = Bands 4% b = lymphocytes with multilobulated nucleus c = N. metamyelocytes 1% N myelocytes 1%  
d = done from other hospital and transfused e = N metamyelocytes 4% N myelocytes 4% f = N metamyelocytes 2% ND = not done.

Hepato- megaly cm	Spleno- megaly cm	Died	Survival, days
8	4	21 4.75	378
palp.	8	13.9 74	26
14	18	5 2.75	30
4	no	29.3.75	14
6	2	25 2.76	144
III	5	15 10 75	18
6	14	3.10 75	4 h
4	no	20 11 76	381
5	palp.	3.5.76	64
no	no	12 6 77	4
no	no	24 2.78	45

10 patients, and in the bone marrow of 7 patients and expressed as percentage calculated from 100 to 200 cell counts.

## Results

### *Incidence Race Sex and Age*

A total of 102 patients were diagnosed as having malignant plasma cell dyscrasia at the University Academic Hospitals of Bloemfontein during the period April 1974 to September 1978. 11 patients (11%) fulfilled the criteria of PCL. The diagnosis was made in all patients with PCL within 3 months from the onset of the first symptoms. The majority of patients (78%) were of negroid origin. This incidence is higher than expected with 59% of Sotho and Tswana blacks in the region. Males were predominantly affected (73%). This is in contrast to findings in plasma cell malignancies in general where the male:female ratio was found to be 1.1 [15] Woodruff *et al.* [36] Prizanski *et al.* [20] Kyle *et al.* [14] and Isobe *et al.* [1] found the male incidence with PCL of 50, 60, 75 and 100%, respectively. No reason for the male incidence is evident, but it may reflect specific race characteristics, or might be due to the small number of patients. The age of our patients varied from 19 to 89 years, with 27% younger than 40 years. Plasma cell malignancy occurs in younger age groups in the negroid population of our milieu, and may be an explanation for this finding (unpubl. observation in the same population group). Patient 6 is the youngest case of PCL reported in the literature.

### *Anaemia*

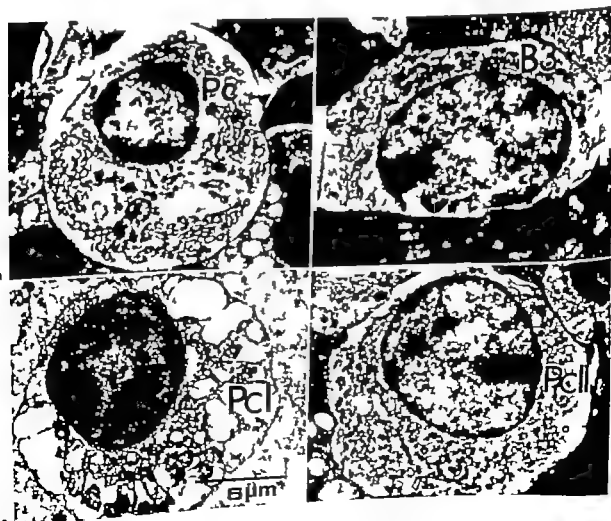
All but one of patients were anaemic. The anaemia was severe (Hb 3-5 g/l) in

Peripheral blood		Bone marrow		TEM	
TEM		LM		TEM	
PCI, %	PCII, %	BLAST %	PC, %	PCI %	PCII, %
92	8		6-7	98	2
79	13	91		90	5
30	65			ND	
10	62		78	ND	
10	22			12	49
	48	98			III
80	13	ND		ND	
	85	95		2	90
68	18			85	5
75	10		95		10
ND			95	ND	

Table III Immunological and biochemical data at admission to the hospital of 11 patients with PCL

Patient No	Total protein, g/l	Albumin	s-Ig			u-Bence-Jones	Paraprotein	s-Ca, mmol/l	s-Urea, mmol/l
			IgG	IgA	IgM				
1	129.0	27.0	770*	5	40	pos/k	IgG/k	2.08	12
2	44.4	29.5	60	10	30	pos/la	LC/la	2.06	14.6
3	86.0	41.6	151	31	57	pos/la	LC/la	2.06	50.3
4	60.1	32.1	157	10	720	neg	neg	2.06	10.2
5	94.4	35.7	438	52	60	neg	IgG/k	1.60	7.6
6	51.0	37	60	10	40	pos/la	LC/la	1.60	14.0
7	5							2.08	50.3
8	61.2	31.8	150	83	135	pos/la	LC/la	2.09	7.3
9	86.0	14.1	1,040	15	10	pos/la	LC/la	2.90	24.0
10	43.4	20.0	60	10	60	pos/k	LC/k	0.80	32.0
11	59.9	48.8	-			neg	neg	2.06	7.1

a = Should be higher cryoglobulins were not taken into consideration b = not done, the patient died 4 h after admission c = non-secretory



45%, moderate (Hb 7–8 g/l) in 55% of the patients and was generally normochromic normocytic (73%) but some cases, with border line cases included (27%) had macrocytic hyperchromic anaemia. We concluded that anaemia in this series of PCL was more severe than found in the other four large reported series 91% of our patients had Hb levels between 3.1 and 7.7 g/l.

#### *Hepatomegaly and Splenomegaly*

Hepatomegaly was present in 82%, splenomegaly in 64% of the patients and 64% of the patients had hepatosplenomegaly. These organs were not enlarged in only 2 patients. The incidence of hepatosplenomegaly is somewhat higher than in the series of Kyle *et al.* [14] where splenomegaly was present in 41% of the patients, hepatomegaly in 53%, hepatosplenomegaly in 35%, and patients exhibiting no enlargement of these organs, 41%.

#### *Bleeding Diathesis*

A bleeding tendency was present in 64% of our patients at the time of presentation. Symptoms were mainly related to gastrointestinal bleeding, but epistaxis, metrorrhagia and echymoses also occurred. This was attributed to thrombocytopenia present in all bleeding patients. Platelet counts varied from 24 to  $108 \times 10^9/l$  in this group of patients. Other studies of haemostasis were not performed. It is possible that other causes may complicate haemorrhage in para-

proteinasaemias [9]. Kyle *et al.* [14] did not record the presence of haemorrhage in their patients, which was present in 50% of the patients studied by Pruzanski *et al.* [20] and in 27% of those studied by Woodruff *et al.* [36]. Pruzanski *et al.* regarded the bleeding tendency as characteristic of PCL, and together with the diffuse bone marrow and peripheral blood involvement they related this disease to acute leukaemia.

#### *Skeletal Disease*

In 4 out of 8 patients where it could be assessed, bone pain was present. Skeletal X-rays were performed on 10 patients: 3 were normal, 4 had osteoporosis and 3 had osteolytic lesions. Osteolytic lesions were widely spread in 1 patient only. The incidence of bone lesions does not differ from that seen in myeloma, although the osteolytic lesions are not as widespread (only 1 out of 10 patients had a such lesion).

#### *Hyperviscosity*

Only 1 patient (No. 1) had the rare hyperviscosity syndrome corresponding to its low incidence in multiple myeloma of 2–4% [35]. This is the only patient reported with hyperviscosity and PCL from the largest available series in this condition.

#### *Protein Studies*

Serum total protein concentrations varied from 43.4 to 129 g/l. In 55% of the patients total protein concentrations were actually lower than normal (43.3–61.2 g/l) and 27% had increased protein values (86.0–129.0 g/l). Albumin levels were frequently low but varied from 14.1 to 48.8 g/l. The immunoglobulin levels were analysed after taking into consideration the different normal values obtained for canceroids (IgG

Fig. 1. Synchronous plasma cell (PC), B lymphocyte in the 3rd transformation stage (B3), Asynchronous plasma cell I (PcI) & Asynchronous plasma cell II (PcII).



100-240 IgA 70-280 IgM males 70-290 and females 120-450 IU/ml) and negroids (IgG 110-320 IgA 80-300 IgM males 100-350 and females 120-450 IU/ml). Increased values of IgG were found in 3 out of 10 patients, normal levels found in 3 out of 10 patients and low levels in 4 out of 10 patients. Increased values of IgA were not observed. Normal values of IgA were found in 2 cases while 8 out of 10 patients gave low levels. Increased IgM levels of the polyclonal type were found in 1 case and reduced levels in 8 out of 10 cases. Thus, IgM immunoglobulins were generally decreased, which is roughly comparable with IgA in this PCL study. Serum paraprotein level in 3 out of 10 patients was of the IgG class, while in 5 patients it was of pure light chain and in 2 out of 10 patients no paraprotein was found. In patients without paraprotein the diagnosis was based on the finding of more than 27% of plasma cells in the peripheral blood, and more than 78% in the bone marrow (table II). Bence Jones proteins were found in the urine of 7 out of 10 patients; the kappa:lambda patients ratio was 2.5. The frequency of the lambda type light chain in this series is significantly higher thus differing from findings reported in the largest series of PCL patients by Kyle *et al.* [14] when out of 7 patients typed, 4 were kappa. In the second largest series of published PCL, the 6 patients typed had a kappa:lambda ratio of 1.1 [36]. When LC of lambda type was associated with amyloid, in the light chain-type of multiple myeloma, it was found to influence the prognosis unfavourably [24]. The investigation for amyloid was not carried out in most of our patients and thus we cannot judge or evaluate the prognostic value of the lambda type paraprotein in this series.

### *Other Haematological Features*

Neutropenia was common (36%) and neutrophilic leucocytosis did not occur. Assessed by light microscopy lymphocytopenia was present in 37% and lymphocytosis in 9% of the patients. Morphological features in MG-G and cytochemistry did not identify the leukaemic cell type in 73% of our patients. In only 27% of patients were the immature cells identified as PC in light microscopy.

### **Discussion**

Peripheral or bone marrow or both of patients with haematological malignancies are almost always examined with TEM in our department. The peripheral blood of 10 out of 11 patients with PCL were examined by TEM additionally 7 of these also had bone marrow examination by the same procedure. The striking feature of this series is the importance of the TEM in PCL. Erroneous diagnoses made if the TEM was not used included acute undifferentiated leukaemia and lymphoproliferative disease, all associated with paraproteinaemia and/or Bence Jones proteinuria. There are numerous reports in the literature of paraproteinaemias and Bence Jones proteinurias associated with acute/chronic leukaemia or lymphoma [1 4 6 8 13 19 22, 25 32, 35, 37 38]. Our experience suggests that some of these cases may have been PCL. However we also have experience with CLL and lymphomas with IgG paraproteinaemia/Bence Jones proteinuria or both with B lymphocyte cell population in TEM [unpubl].

Graham and Bernier [10] introduced the diagnostic and prognostic value of the asyn-

chronous plasma cells found in 50 out of 52 of their patients with plasma cell malignancies. Asynchronous plasma cells are considered diagnostic for plasma cell malignancy [10-27] PCI and PCII (in the *Graham and Bernier's* nomenclature these are called markedly or moderately asynchronous plasma cells, respectively) were not found in 244 bone marrows of patients with diseases other than malignant plasma cell dyscrasia [27]. In cases of malignant plasma cell dyscrasia PCI and PCII were always present although there may be an admixture with normal PC [27]. The PCI was the sole or dominant type of plasma cell in 5 out of 10 patients in this series, while in 5 patients the PCII was the dominant finding. In multiple myeloma [personal, unpubl. experience] only 5 out of 41 patients had the sole or predominant type of cell of the PCL. We related findings from this study to the patients survival in analogy to what was done in studies of other plasma cell malignancies [10, 36]. There was no obvious morphological plasma cell type predominance in the long survivors (relatively) from this series with PCL (patients 1-5 and 8, table I-II).

9 patients were treated with melphalan and glucocorticoids as described elsewhere [3]. Objective remission, as assessed by the decrease in paraprotein, improvement in bone marrow function and clinical symptoms, were seen in 3 patients (33%). Remissions were of short duration and the survival of these 3 patients was 381, 379 and 144 days, respectively. The number of patients in this series is small and no influence of paraprotein, immunoglobulin class, light chain type, Bence Jones proteinuria, serum calcium, serum urea, extent of skeletal lesions or plasma cell type (PCI or PCII) on the clinical course was evident. The mean

survival was 3.3 months. The disease thus has a much poorer prognosis than multiple myeloma which on similar treatment schedule has a mean survival of 34 months [2]. Evidently alternative treatment protocols should be investigated for PCL.

In conclusion, in our series PCL emerges as a distinct clinical entity: patients are severely anaemic, hepatosplenomegaly is prominent, bone lesions are not usually osteolytic and the response to treatment with alkylating agents and glucocorticoids is poor. The diagnosis is difficult since the circulating plasma cells may have morphological features which only allows the diagnosis to be made after TEM examination.

If the peripheral blood of cases of acute leukaemias and immunocytic dyscrasias is routinely examined by TEM, PCL appears to be a common variant of malignant plasma cell dyscrasias - it was 11% in this series.

### Acknowledgement

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100–240 IgA 70–280 IgM males 70–290 and females 120–450 IU/ml) and negroids (IgG 110–320 IgA 80–300 IgM males 100–350 and females 120–450 IU/ml) Increased values of IgG were found in 3 out of 10 patients, normal levels found in 3 out of 10 patients and low levels in 4 out of 10 patients. Increased values of IgA were not observed Normal values of IgA were found in 2 cases while 8 out of 10 patients gave low levels. Increased IgM levels of the polyclonal type were found in 1 case and reduced levels in 8 out of 10 cases Thus IgM immunoglobulins were generally decreased which is roughly comparable with IgA in this PCL study Serum paraprotein level in 3 out of 10 patients was of the IgG class, while in 5 patients it was of pure light chain and in 2 out of 10 patients no paraprotein was found In patients without paraprotein the diagnosis was based on the finding of more than 27% of plasma cells in the peripheral blood, and more than 78% in the bone marrow (table II) Bence Jones proteins were found in the urine of 7 out of 10 patients the kappa lambda patients ratio was 2.5 The frequency of the lambda type light chain in this series is significantly higher thus differing from findings reported in the largest series of PCL patients by Kyle *et al* [14] when out of 7 patients typed, 4 were kappa. In the second largest series of published PCL, the 6 patients typed had a kappa lambda ratio of 1.1 [36] When LC of lambda type was associated with amyloid, in the light chain-type of multiple myeloma, it was found to influence the prognosis unfavourably [24] The investigation for amyloid was not carried out in most of our patients and thus we cannot judge or evaluate the prognostic value of the lambda type paraprotein in this series.

### Other Haematological Features

Neutropenia was common (36%) and neutrophilic leucocytosis did not occur Assessed by light microscopy lymphocytopenia was present in 37% and lymphocytosis in 9% of the patients. Morphological features in MG-G and cytochemistry did not identify the leukaemic cell type in 73% of our patients. In only 27% of patients were the immature cells identified as PC in light microscopy

### Discussion

Peripheral or bone marrow or both of patients with haematological malignancies are almost always examined with TEM in our department. The peripheral blood of 10 out of 11 patients with PCL were examined by TEM additionally 7 of these also had bone marrow examination by the same procedure The striking feature of this series is the importance of the TEM in PCL. Erroneous diagnoses made, if the TEM was not used included acute undifferentiated leukaemia and lymphoproliferative disease, all associated with paraproteinaemia and/or Bence Jones proteinuria. There are numerous reports in the literature of paraproteinaemias and Bence Jones proteinurias associated with acute/chronic leukaemia or lymphoma [1 4 6 8 13 19 22, 25 32, 35, 37 38] Our experience suggests that some of these cases may have been PCL. However we also have experience with CLL and lymphomas with IgG paraproteinaemia/Bence Jones proteinuria or both with B lymphocyte cell population in TEM [unpubl.]

Graham and Bernier [10] introduced the diagnostic and prognostic value of the asyn-

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## Material and Methods

Blood samples from 1 homozygote and 3 heterozygotes for HPFH Negro type belonging to the same family were examined. The samples, collected in Ghana, were shipped airmail to Torino.

Hemoglobin electrophoresis was performed on Cellulose acetate Tris-glycine buffer at pH 8.6 [13]. Hb F quantitation was obtained both by means of the alkali denaturation method [4] and by chromatography on DEAE-cellulose, according to Abraham *et al.* [1]; this technique was also utilized to separate Hb F from the hemolysates. After tryptic digestion of the whole globin F and/or the purified  $\gamma$  chains [16], peptide maps were obtained at pH 6.4 as described by Sack *et al.* [19], in order to investigate the presence of Ty chains, as previously described [14].

The  $\gamma$  136 glycine/alanine ratio was determined after cleavage of 10 mg whole globin F with 100-fold molar excess of CNBr in 70% formic acid at room temperature for 24 h. CNBr was eliminated by vacuum drying and the residue was fingerprinted on paper at pH 6.4 in order to separate the  $\gamma$  CB3 fragment as described by Bernal *et al.* [3] and by Komazawa *et al.* [11]. Amino acid analysis was performed on an LKB mod. 4101 amino acid analyzer after elution of the peptides directly from the paper with 6 N HCl and their hydrolysis in vacuum at 110 °C for 4 h. The  $\alpha/\gamma$  ratio was also evaluated by means of the new technique of  $\gamma$ -chain separation on electrophoresis [16]. Electrophoresis was performed on cellulose acetate using modification of Bernal's

unpublished technique [personal communication]: globin, dissolved in electrophoresis buffer (6 M urea, 0.1 M Tris-H<sub>2</sub>PO<sub>4</sub> pH 7.2, 10 mM EDTA, 3% MP-40, 1/2-mercaptoethanol) were run for 16 h, 100 V 22 °C. At the end of the run, proteins were stained with Amido-Schwartz and quantitated by means of densitometer (Superfantom CGA).

## Results

The family pedigree is depicted in figure 1. Hb F makes up all of the propositus hemoglobin, normal Hb A and Hb A<sub>2</sub> being absent. The mother (I 2) and the brother (II 2) have about 25% Hb F; the sister has about 32%.

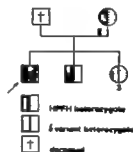


Fig. 1. Family pedigree.

Table I. Results of  $\gamma$ -chain composition studies

	Hb F %	Gly ( $\gamma$ CB3)	Ala ( $\gamma$ CB3)	Gly %    Ala % (cellulose acetate electrophoresis)		$\gamma$ chains
I-1 Mother	32	0.58	2.50	48	52	d.
II-1 Propositus	100	0.63	2.37	70	30	d.
II-2 Sister	25	0.43	2.61	39	61	d.
II-3 Brother	24	0.53	2.48			d.

Glycine and alanine ratios are expressed as number of residues present in the  $\gamma$  CB3 fragment

d. = Not detected



## Analysis of the $\gamma$ Chains in a Homozygote for HPFH Negro Type and in Three Related Heterozygotes

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**Key Words.** Hb F  $\gamma$ -Chain composition HPFH Negro type

**Abstract.** The  $\gamma$ -chain structure of a homozygote for HPFH Negro type previously described by Acquave and co-workers, and of 3 heterozygotes of the same family has been evaluated.  $^0\gamma$  and  $^1\gamma$  chains were present in a ratio of fetal type (7/3) in the homozygote and in ratios of 2/3 and 1/1 in the heterozygotes, in agreement with data of the literature.  $^2\gamma$  chains were absent in all the cases. Our results compared with those previously reported for Negro and Greek HPFH suggest that the  $^2\gamma$  gene is never linked with the HPFH determinant.

### Introduction

Since it has been demonstrated that  $\gamma$  chains of human fetal hemoglobin differ for the presence of either an alanine or a glycine residue at position 136 [17] the HPFH Negro type has been classified into two groups: one with only  $^0\gamma$  and another with both  $^0\gamma$  and  $^1\gamma$  chains in different proportions [20].

In 1975 a new  $\gamma$  variant (Hb F Sardinia) [6] carrying threonine instead of isoleucine in position 75 was described; subsequently, the same  $\gamma$  chain ( $^2\gamma$  chain) was found in a high percentage of Caucasian subjects [14] and with lower incidence also in Negro people [10, 18]. Recently the  $^2\gamma$  chain has

been documented to be an allelic variant of the  $^1\gamma$  chain both by means of statistical analysis of a wide series of samples [18] and by direct examination of fingerprints of purified  $^1\gamma$  chains [16]. As reported by Schroeder et al. [18] the  $^2\gamma$  chain seems to be absent both in homozygous and in heterozygous HPFH Negro type.

In order to clarify the mechanism of the  $\gamma$  gene expression in HPFH Negro type, we have studied the  $\gamma$  chain composition in a homozygote for this condition and in 3 heterozygotes of the same family. The proband was firstly examined by Acquave et al. [2] and his glycine/alanine ratio at position 136 was already studied by Ringelhan et al. [15].

On the other hand,  $\gamma$  chains seem to be absent even in other types of HPFH. In fact, we could not find  $\gamma$  in 3 heterozygotes for HPFH Greek type [5] and in 4 Italian subjects from another family showing about 10% Hb F homogeneously distributed among the erythrocytes and entirely of the  $\gamma$  type [unpublished data]. Also Schroeder *et al.* [18] were unable to detect  $\gamma$  chains in 3 Caucasian carriers of HPFH  $\gamma$  type.

Even if the cases up to now examined are still limited, these data suggest that the  $\gamma$  gene is never linked to the HPFH determinant and seem to exclude a  $\gamma$ -chain production in trans.

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In I 2 and II 3 normal Hb A<sub>2</sub> was not detected, but a slow moving fraction (about 2%) was present even if it could not be identified because of lack of material it was interpreted as an Hb A<sub>2</sub> variant.

Table I reports the  $\gamma$  chain composition of all the subjects examined the  $\alpha\gamma/\gamma$  ratio at position 136 of the propositus was of fetal type (about 7/3) in the mother and in the brother it was about 1/1 and in the sister about 2/3  $\gamma$  chains were absent in all the cases.

### Discussion

Up to now several subjects, both heterozygous and homozygous for HPFH Negro type, have been studied with regard to the  $\gamma$  chain composition [7-15] however only in a few cases complete family studies were carried out in order to evaluate the inheritance of the  $\gamma$  gene expression [7].

In the family described in this paper the father is deceased however the finding of a  $\delta$  variant allowed us to ascertain the genetic pattern of this subject. In fact, the mother showed both the HPFH and the  $\delta$  variant since the  $\delta$  gene linked to the HPFH Negro type has been demonstrated to be deleted [12] the  $\delta$  variant in this subject must be on the homologous chromosome consequently the daughter who has inherited the  $\delta$  variant from the mother must have the paternal HPFH thus, we may assume to have examined both the paternal and the maternal HPFH.

The presence of a  $\delta$  variant in association with the HPFH Negro type has also been reported by Huisman *et al* [9] in a black family.

The  $\alpha\gamma/\gamma$  ratio in the mother is very sim-

ilar to that observed in the brother (about 1/1) while in the sister who has the paternal HPFH more  $\gamma$  chains have been found ( $\alpha\gamma/\gamma = 2/3$ ). The  $\alpha\gamma/\gamma$  ratio observed in the homozygote in our laboratory is about 7/3 and is in keeping with the data obtained in the same subject by Ringelhan *et al* [15] it must be underlined that all the homozygotes up to now studied present a  $\alpha\gamma/\gamma$  ratio of fetal type [15] always higher than that reported in the heterozygotes. In particular in family M., described by Huisman *et al* [7] the homozygote produced more  $\gamma$  chains than the heterozygotes from the maternal and the paternal side.

While in  $\beta$ -thalassaemia it has been observed that the  $\alpha\gamma/\gamma$  ratio is generally intermediate between the values of the parents [8] this fact does not happen in the HPFH Negro type why the  $\gamma$ -chain composition varies between the homozygotes and the heterozygotes is still obscure possibly as suggested by Huisman *et al* [7] an unknown factor alters the expression of the gene in HPFH.

As far as the problem of the  $\gamma$  chain is concerned, we could not find this type of chain in any subjects examined (table I), neither in the total Hb F nor in separated  $\gamma$ -chain. While the  $\gamma$  detection on finger prints of the total Hb F may be uncertain when this chain is present in low percentage [14] the quantitation of  $\gamma$  chain in purified  $\gamma$  chain is much more reliable the absence of  $\gamma$  chain in our cases confirms the previous data by Schroeder *et al* [18] who could not show this type of chain in a wide series of carriers of HPFH Negro type. The fact that all the subjects up to now examined are negative for  $\gamma$  chain might be related to the low incidence of this chain in black people [10-18].

## Haemoglobin D Los Angeles, D- $\beta^+$ Thalassaemia, and D- $\beta^0$ -Thalassaemia

### A Report of Two Canadian Families

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**Key Words.**  $\beta^+$  Thalassaemia  $\beta^0$  Thalassaemia Hb D Los Angeles

**Abstract.** The first 2 Canadian cases of combinations of Hb D Los Angeles and  $\beta$ -thalassaemia were found in an Italian family and an East Indian family in Hamilton, Ont. The structure of the Hb D variant was identified by radioimmunoassay. The  $\beta^+$ -thalassaemia trait in the Italian family and the  $\beta^0$ -thalassaemia trait in the East Indian family were confirmed by studies of globin chain synthesis. Previous cases of Hb D- $\beta$ -thalassaemia were summarized and family studies of the 2 new cases were presented.

Haemoglobin D Los Angeles ( $\alpha_2\beta_2$ ,  $\alpha_2\beta_2$ ) was first discovered by *Itano* [18] and its structure elucidated by *Baglioni* [3]. It is also known as Hb D D Punjab, D North Carolina, D Chicago, D Portugal, and D Oak Ridge. Besides being found in heterozygotes and homozygotes, it has also been found in association with  $\alpha$ -thalassaemia [6] and  $\beta$ -thalassaemia (see 'Discussion'). Recently 2 cases of double heterozygosity for Hb D Los Angeles and  $\beta$ -thalassaemia have been found in Hamilton, Ont.: 1 in an Italian woman with Hb D and  $\beta^+$ -thalassaemia (family S) and 1 in an East Indian woman with Hb D and  $\beta^0$ -thalassaemia (family M). This paper reports the haematological data

and the studies of globin chain synthesis in these two families.

### Material and Methods

Blood was collected in EDTA vacutainers. Haematological evaluation was made by standard laboratory methods [9].  $\alpha/\beta$ -Chain synthesis ratio was determined as described by *Halowan and Jowett* [16] using  $^3\text{H}$ -leucine. Haemoglobin analysis was made by starch gel electrophoresis at pH 9 using Tris-borate-HCl buffer [11]. Hb A<sub>2</sub> was determined by microcolumn chromatography [12] and quantitative analysis of the other haemoglobin fractions by DEAE-cellulose column chromatography [1]. Hb F was measured by the Betke method [5] and free erythrocyte protoporphyrin by

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Hb F <sup>a</sup> %	Hb A <sub>2</sub> <sup>a</sup> %	Hb D <sup>a</sup> %	Hb A %	Relative $\beta/\alpha$ ratio <sup>a</sup>
0.9	4.3 <sup>a</sup>	82.0	12.8	0.73 <sup>b</sup>
1.3	2.7 <sup>a</sup>	43.1	52.9	0.95
1.3	5.2	0	93.5	0.43
0.7	3.1	0	96.2	0.97
1.1	5.8	0	93.1	0.30
1.4	6.5	0	92.1	0.30
0.7	4.1	95.2	0	0.47
0.5	2.8	0	96.7	n.d.
17.5	3.7	0	78.8	d.

posita, together with the elevated Hb A<sub>2</sub> level and the absence of Hb A was compatible with a case of Hb D- $\beta^0$ -thalassaemia. Family studies indicated that the older girl inherited the Hb D trait and the 3-month-old baby inherited the  $\beta^0$ -thalassaemia trait from the mother.

#### Synthesis Studies

Carboxymethyl-cellulose column chromatographic separation of radioactive globin chains from the peripheral blood of the two proposita<sup>a</sup> showed the presence of both  $\beta^0$  and  $\beta^A$ -chains in the Italian proposita but only  $\beta^0$ -chains in the Indian proposita. Table I lists the  $\alpha/\beta$  ratios of all the available members of both families.

#### Identification of the Hb D Variants

Haemoglobin 3 was excluded from both proposita<sup>a</sup> by the negative Itano solubility test and electrophoretic patterns on acid-citrate agar. Radioimmunoassay was performed in Augusta, Ga., and confirmed Hb D Los Angeles in both proposita<sup>a</sup>.

phoresis showed one major Hb band (95.2%) in the D position and Hb A<sub>2</sub> (4.1%). There was no Hb A. The hypochromic and microcytic picture of this pro-

#### Discussion

Since its discovery in 1951 and its identification in 1962, Hb D Los Angeles has

Fig. 1. Pedigree of family 3 and family M.

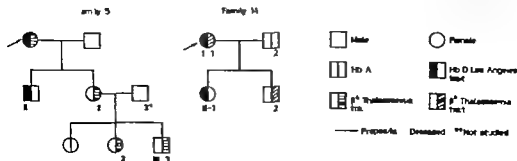


Table 1 Haematological and analytical data of family S and family M

Case	Hb type	Age years	Hb g/dl	REC 10 <sup>12</sup> /l	MCV fl	MCH pg	Reticulo- cytes, %	FEP g/dl RBC
<i>Family S</i>								
I 1	D- $\beta$ -thal	55	13.8	6.45	61	21.3	6.7	72
II 1	AD	30	15.9	5.23	87	30.9	1.2	53
II 2	A- $\beta^+$ -thal	33	11.6	5.33	62	21.8	5.6	72
III 1	AA	13	13.5	4.70	79	28.3	1.5	47
III-2	A- $\beta^+$ -thal	10	11.1	5.66	57	19.7	3.2	63
III 3	A- $\beta$ -thal	9	12.3	5.99	58	20.7	2.4	54
<i>Family M</i>								
I 1	D- $\beta^+$ -thal	19	9.7	4.52	68	21.6	4.4	35
I 2	AA	25	14.6	5.09	85	27.0	1.2	21
II 1	AD <sup>a</sup>	2						
II 2	A- $\beta^+$ -thal	3/12	8.7	4.07	65	21.9	n.d.	n.d.

By a spectrofluorometric method (normal 20–80  $\mu$ g/dl RBC).

<sup>b</sup> By Betke's method (adult normal 0.4–1.2%).

By DEAE-cellulose microcolumn chromatography (normal 1.9–3.3%).

<sup>c</sup> By DEAE-cellulose analytical column chromatography

Adult normal range 0.94–1.10.

<sup>d</sup>  $\beta^A + \beta^D/a$  ratio, average of duplicated tests.

<sup>e</sup>  $\beta^D/a$  ratio,  $\beta^A$  peak was absent in chromatogramme.

<sup>f</sup> By starch gel electrophoretic pattern. Haematological data were not obtainable because of laboratory accident.

spectrofluorometric method [23]. Radioimmunoassay was used for the identification of the Hb D variant [13].

## Results

### Haematological and Analytical Studies

The pedigree, haematological and analytical data of both families are given in figure 1 and table I. The probanda of family S is a 55 year-old Italian woman who has a son a daughter and four grandchildren. Haematological evaluation revealed a hypochromic and microcytic red cell morphology. Her free erythrocyte protoporphyrin level was normal. Starch gel electrophoresis showed a major Hb band (82.0%) in the D

position, Hb A (12.8%) and Hb A<sub>2</sub> (4.3%). The hypochromic and microcytic picture of the probanda, together with the elevated Hb A<sub>2</sub> level and the presence of Hb A was compatible with a case of Hb D- $\beta^+$ -thalassaemia. Family studies showed that the son inherited the Hb D trait and the daughter inherited the  $\beta^+$  thalassaemia trait from their mother. Two of the three grandchildren were also heterozygous for  $\beta^+$ -thalassaemia.

The probanda of family M is a 19-year old East Indian woman who is married and has a 2-year-old girl and a 3-month-old baby boy. She was mildly anaemic with a haemoglobin level of 9.7 g/dl. Her red cell morphology showed hypochromia and microcytosis. The free erythrocyte protoporphyrin level was normal. Starch gel electro-

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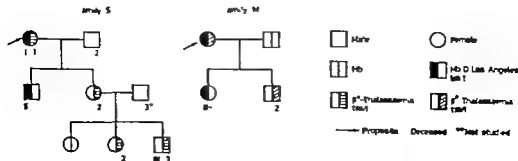




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been found in 'white Americans [2] 'black Americans [8] American Indians [15] East Indians [7] Thais [31] Filipinos [29] Turks [22] Greeks [10] Italians [21] Bulgarian Jews [24] Dutch [14] and Canadians [30] Combinations of Hb D and  $\beta$ -thalassaemia traits have also been documented. The first case of Hb D- $\beta$ -thalassaemia was found in a Persian girl in 1956 [17] Since then, 18 cases of D- $\beta$ -thalassaemia [4 10 17 19-21 24 25 27 28] and 1 case of D- $\beta$ -thalassaemia have been reported [26] The D variant in 3 of these cases was identified as Hb D Los Angeles by chemical analysis [24-26] Clinical manifestations of the double heterozygotes varied from normal to anaemia with hepatosplenomegaly with or without elevated Hb F

This paper reports the first two Canadian families in which Hb D Los Angeles and  $\beta$ -thalassaemia traits were found. The Italian family is the second family described in the literature with the combination of Hb D and  $\beta^+$  thalassaemia [26] The Indian family has the more frequent combination of Hb D and  $\beta^0$  thalassaemia. Thalassaemia traits in these two families were initially detected by the hypochromic and microcytic red cell morphology and elevated Hb A<sub>2</sub> levels. Iron deficiency was excluded by the normal free erythrocyte protoporphyrin levels. The presence of 12.8% Hb A in the former proposita who had no previous transfusion indicated the presence of a  $\beta^+$  thalassaemia gene in the Italian family. The absence of Hb A in the latter proposita indicated the presence of a  $\beta^0$ -thalassaemia gene in the Indian family. These observations were supported by a  $\beta^D + \beta^A/a$  ratio of 0.72 in the Italian proposita and a  $\beta^D/a$  ratio of 0.47 in the Indian proposita. The abnormal Hb D variants in these two families were identified

to be Hb D Los Angeles by a more recent radioimmunological technique [13] This method enables the structural characterization of the common haemoglobin variants without resorting to time-consuming chemical analysis. Clinically besides mild reticulocytosis in family S and mild hypochromic anaemia in family M, both propositae were asymptomatic.

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France) gradient [5]. Examination of the separated cells showed 90-99% mononuclear cells, of which less than 5% were monocytes.

Spontaneous rosette formation with sheep erythrocytes (E rosettes) and rosette formation with erythrocyte-antibody-complement complexes (EAC rosettes) were determined as described by Beck [1] and Sjöström *et al.* [20], respectively. A mononuclear cell with three or more adherent erythrocytes was considered to be rosette. At least 200 viable cells were counted in a hemacytometer chamber. Smears were prepared and May-Grunwald-Giemsa stained to assess the morphology of the rosette-forming cell. Each test was made in duplicate.

The white blood cell total and differential counts were simultaneously determined to permit the calculation of the absolute number of circulating B- and EAC-rosette-forming cells.

Phytohemagglutinin (PHA) transformation at 72 h was expressed morphologically as the percentage of transformed lymphocytes after counting at least 200 lymphocytes in each preparation on May-Grunwald-Giemsa stained slides. As a transformed lymphocyte was considered larger cell with lower nuclear-cytoplasmic ratio a vacuolated and more basophilic cytoplasm and lighter and more granular nucleus often with identifiable nucleoli. Each sample was suspended in duplicate with PHA (Wellcome, HA15) and without PHA [5].

Normal values were obtained by performing the tests described above in 20 healthy adults.

#### Skin Tests

The skin test antigens used included *Candida* (1:10), trichophyton (1:10), PPD (2 TU) and PHA (0.1 mg/0.1 ml; Purified Phytohemagglutinin, Wellcome). All antigens were applied by intradermal injection in volume of 0.1 ml through 27 gauge needle. Reactions were read at 48 h by measuring the indurated area as its perpendicular diameters, and the mean diameter of induration for each test was determined. Any test, except PHA skin test, that measured 5 mm or more in average diameter was considered positive reaction. Results of PHA skin testing of the controls ranged from 15 to 60 mm, with mean of 33 mm. Taking into account these normal values, PHA skin test reaction was considered positive if an induration greater than 10 mm was observed.

Patients were considered reactive if they were responsive to at least one antigen. When battery of three or more skin tests was used, patients unresponsive to all tested antigens were evaluated as anergic, but if only one or two skin tests were performed, patients unresponsive to the tested antigens were considered relatively anergic [11].

The control group consisted of 18 healthy adult subjects who received the entire battery of four skin tests.

#### Statistical Analysis

Differences between groups were evaluated with Student's *t* test or the chi square test ( $\chi^2$ ) using Yates correction.

#### Results

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Patients with sickle cell anaemia (SCA) are highly susceptible to infections, a finding attributed to a number of factors [6, 12, 14, 21]. The defect of immunologic function in SCA has been reported to be similar to that described in asplenic individuals [18, 19]. Defective immune function, particularly depressed cellular immunity, has been documented in many human diseases [4, 13]. Our study evaluates selected cellular immune parameters in a series of patients with SCA as another step in further investigation of SCA host defenses against infections.

### Material and Methods

The study population consisted of 20 outpatients (14 men and 5 women) with homozygous SCA from the clinical Haematology Adult Service at the Institute of Haematology and Immunology of Havana, Cuba. Their ages ranged from 17 to 44 years, with a mean age of 27 years. All patients were in a relatively good state of health, and none had suffered from any form of crisis in the month preceding the study.

#### *Lymphocyte Studies*

Venous blood from the test subjects was heparinized and lymphocytes were separated on a Ficoll-Telebreix (Laboratoires Andre Guertel,

France) gradient [3]. Examination of the separated cells showed 90-99% mononuclear cells, of which less than 5% were monocytes.

Spontaneous rosette formation with sheep erythrocytes (E rosettes) and rosette formation with erythrocyte-antibody-complement complexes (EAC rosettes) were determined as described by Bach [1] and Stjernwåhl *et al.* [20], respectively. A mononuclear cell with three or more adherent erythrocytes was considered to be a rosette. At least 200 viable cells were counted in a hemacytometer chamber. Smears were prepared and May-Grienswald-Giemsa stained to assess the morphology of the rosette-forming cell. Each test was made in duplicate.

The white blood cell total and differential counts were simultaneously determined to permit the calculation of the absolute number of circulating E- and EAC-rosette-forming cells.

Phytohemagglutinin (PHA) transformation at 72 h was expressed morphologically as the percentage of transformed lymphocytes after counting at least 200 lymphocytes in each preparation on May-Grienswald-Giemsa stained slides. A transformed lymphocyte was considered larger cell with lower nuclear-cytoplasmic ratio, vacuolated and more basophilic cytoplasm and a lighter and more granular nucleus often with identifiable nucleoli. Each sample was suspended in duplicate with PHA (Wellcome, HA15) and without PHA [5].

Normal values were obtained by performing the tests described above in 20 healthy adults.

#### Skin Tests

The skin test antigens used included *Candida* (1:10), trichophyton (1:10), PPD (2 TU) and PHA (0.1 mg/0.1 ml<sup>2</sup>). Purified Phytohemagglutinin, (Wellcome). All antigens were applied by intradermal injection in a volume of 0.1 ml through 27-gauge needle. Reactions were read at 48 h by measuring the indurated area in two perpendicular diameters, and the mean diameter of induration for each test was determined. Any test, except PHA skin test, that measured 5 mm or more in average diameter was considered positive reaction. Results of PHA skin testing of the controls ranged from 15 to 60 mm, with mean of 33 mm. Taking into account these normal values, a PHA skin test reaction was considered positive if an induration greater than 10 mm was observed.

Patients were considered reactive if they were responsive to at least one antigen. When battery of three or more skin tests was used, patients unresponsive to all tested antigens were evaluated as anergic, but if only one or two skin tests were performed, patients unresponsive to the tested antigens were considered relatively anergic [11].

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Venous blood from the test subjects was heparinized and lymphocytes were separated on a Ficoll-Teletex (Laboratoire Andre Guerbert,

Table II. Skin testing in SCA

Skin test antigens	SCA patients			Normal controls			
	total skin tests	positive		total skin tests	positive		
		number	%		number	%	
PHA	11	7	64	18	18	100	< 0.05
PPD	14	5	36	18	12	67	NS <sup>1</sup>
Trichophyton	11	6	55	18	15	83	NS
Candida	13	8	62	18	13	72	NS

NS = Not significant.

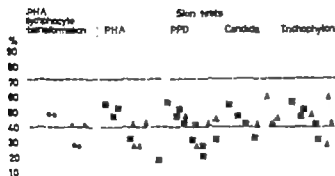


Fig. 1. Correlation between PHA lymphocyte transformation and skin test reactivity.  $\Delta$  = Positive;  $\square$  = negative. — = Normal range of PHA lymphocyte transformation.

relate with PHA-induced lymphocyte transformation (Fig. 1).

### Discussion

Leucocytosis is a frequent finding in SCA. Generally leucocytes range between  $10 \times 10^9/l$  and  $20 \times 10^9/l$ , but the values may be greater [22]. This leucocytosis may explain the slight absolute lymphocytosis found in the studied SCA group.

T lymphocytes, although decreased in percentage in 30% of cases, showed normal absolute values in most patients. Impair-

ment of cell-mediated immunity was found in a high proportion of patients with SCA as assessed by lymphocyte transformation and skin sensitivity. Only half the patients showed normal lymphocyte transformation in response to PHA, and in some cases cutaneous anergy (in skin tests) was observed. Patients with SCA were less responsive to PHA than to the other intradermal skin tests. The study of these immunologic parameters in children with SCA could contribute to a better understanding of the immune system of SCA patients. The dissociation between skin tests and PHA transformation seems paradoxical, but there is evi-

Table I. Immunologic data in SCA

Case No.	WBC $\times 10^9/l$	Lymphocytes		E rosettes		EAC rosettes		PHA	Skin test <sup>b</sup>
		%	$\times 10^9/l$	%	$\times 10^9/l$	%	$\times 10^9/l$		
1	9.5	10	1.0	71	0.7	38	0.4	45	4/4
2	10.5	27	2.9	72	2.0	19	0.5	31	1/2
3	9.7	31	3.0	46	1.4	10	0.3	18	0/1
4	10.1	82	5.3	65	3.4	21	1.1	56	0/4
5	13.6	30	4.1	60	2.4	23	0.9	48	0/4
6	12.1	41	5.0	70	3.5	23	1.1	52	1/4
7	10.5	25	2.6	57	1.5	25	0.7	48	3/3
8	8.8	43	3.8	68	2.6	12	0.5	42	0/3
9	11.5	27	3.1	33	1.0	16	0.5	32	0/4
10	10.8	33	3.6	89	3.2	23	0.8	40	2/3
11	15.7	18	2.8	83	2.4	24	0.7	28	3/3
12	8.7	31	2.7	70	1.9	27	0.7	28	3/4
13	4.4	28	1.2	74	0.9	10	0.1	20	1/2
14	10.6	41	4.3	65	2.8	20	0.9	58	4/4
15	4.9	16	0.8	64	0.5	27	0.2	40	4/4
16	13.3	38	5.0	36	1.8	18	0.9	26	NT <sup>a</sup>
17	8.4	20	1.7	44	0.7	38	0.6	21	NT
18	17.3	19	3.3	50	1.6	30	1.0	23	NT
19	13.7	35	4.8	56	2.7	18	0.9	48	NT
20	12.0	35	4.2	42	1.8	14	0.6	20	NT
Mean $\pm$ SD	10.8 $\pm$ 3.1	30 $\pm$ 10.3	3.3 $\pm$ 1.3	60 $\pm$ 15.1	1.9 $\pm$ 0.9	22 $\pm$ 7.8	0.7 $\pm$ 0.2	36 $\pm$ 13	
<i>Controls</i>									
Mean $\pm$ SD	6.6 $\pm$ 1.1	35 $\pm$ 7	2.4 $\pm$ 0.2	63 $\pm$ 5	1.3 $\pm$ 0.4	25 $\pm$ 6	0.6 $\pm$ 0.2	56 $\pm$ 7.6	
Range	5-10	16-46	1.2-3.7	55-75	0.7-2.3	10-33	0.3-1.1	40-72	

Lymphocyte transformation response to phytohemagglutinin.

<sup>b</sup> Number of positive skin tests/number of used antigens.

NT = Not tested.

50% of the patients. SCA patients showed a mean PHA transformation of 36% compared with 56% in the normal group ( $p < 0.01$ ). In 6 patients (cases No 3 9 16, 17 18 and 20), E rosettes and PHA transformation were simultaneously decreased.

At least one skin test antigen was positive in 10 of 15 patients (67%). If one considers only those 12 patients who received

three or four antigens, 4 of them (33%) were anergic (table I).

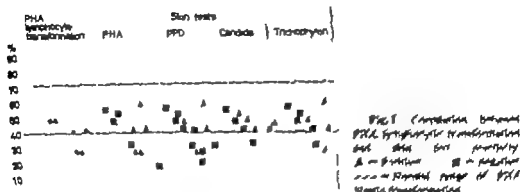
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T lymphocytes, although decreased in percentage in 30% of cases, showed normal absolute values in most patients. Impair

ment of cell-mediated immunity was found in a high proportion of patients with SCA as assessed by lymphocyte transformation and skin tests (only half the patients showed normal lymphocyte transformation in response to PPD and in some cases for candida antigen to skin test as well). Patients with SCA were less responsive to PHA than to the other immunologic skin tests. The study of T cell immunologic parameters in children with SCA as well as adults is a better understanding of the immune system of SCA patients. The observation that some SCA patients manifest immune paradoxes (but that is not

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Mean  $\pm$  SD    10.8  $\pm$  3.1    30  $\pm$  10    3.3  $\pm$  1.3    60  $\pm$  15    1.9  $\pm$  0.9    22  $\pm$  7.8    0.7  $\pm$  0.2    36  $\pm$  13

#### Controls

Mean  $\pm$  SD    6.6  $\pm$  1.1    35  $\pm$  7    2.4  $\pm$  0.2    63  $\pm$  5    1.5  $\pm$  0.4    25  $\pm$  6    0.6  $\pm$  0.2    56  $\pm$  7.6

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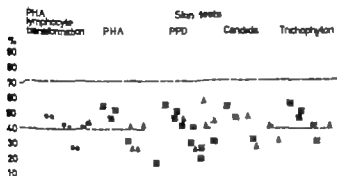


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Mean  $\pm$  SD     $10.8 \pm 3.1$      $30 \pm 10$      $3.3 \pm 1.3$      $60 \pm 15$      $1.9 \pm 0.9$      $22 \pm 7.8$      $0.7 \pm 0.2$      $36 \pm 13$

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Mean  $\pm$  SD     $6.6 \pm 1.1$      $35 \pm 7$      $2.4 \pm 0.2$      $63 \pm 5$      $1.3 \pm 0.4$      $25 \pm 6$      $0.6 \pm 0.2$      $56 \pm 7.6$

Range    5-10    16-46    1.2-3.7    55-75    0.7-2.3    10-33    0.3-1.1    40-72

Lymphocyte transformation response to phytohemagglutinin.

<sup>b</sup> Number of positive skin tests/number of used antigens.

NT = Not tested.

50% of the patients. SCA patients showed a mean PHA transformation of 36% compared with 56% in the normal group ( $p < 0.01$ ). In 6 patients (cases No 3 9 16, 17 18 and 20), E rosettes and PHA transformation were simultaneously decreased.

At least one skin test antigen was positive in 10 of 15 patients (67%). If one considers only those 12 patients who received

three or four antigens, 4 of them (33%) were anergic (table I).

Table II shows the results of skin testing of SCA patients and the control group. Patients with SCA were less reactive to individual skin tests but as can be seen, reactivity was significantly different only with the PHA skin test.

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Table II. Skin testing in SCA

Skin test antigens	SCA patients				Normal controls				P
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		number	%		number	%			
PHA	11	7	64	18	18	100	< 0.05		
PPD	14	5	36	18	12	67	NS <sup>1</sup>		
Trichophyton	11	6	55	18	15	83	NS		
Candida	13	8	62	18	11	72	NS		

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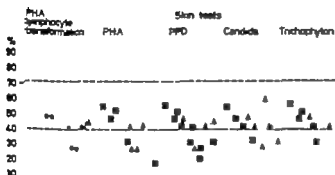


Fig. 1. Correlation between PHA lymphocytic transformation and skin test reactivity.  $\Delta$  = Positive;  $\blacksquare$  = negative. --- = Normal range of PHA blastic transformation.

relate with PHA-induced lymphocyte transformation (Fig. 1).

### Discussion

Leucocytosis is a frequent finding in SCA. Generally leucocytes range between  $10 \times 10^9/l$  and  $20 \times 10^9/l$ , but the values may be greater [22]. This leucocytosis may explain the slight absolute lymphocytosis found in the studied SCA group.

T lymphocytes, although decreased in percentage in 30% of cases, showed normal absolute values in most patients. Impair

ment of cell-mediated immunity was found in a high proportion of patients with SCA as assessed by lymphocyte transformation and skin sensitivity. Only half the patients showed normal lymphocyte transformation in response to PHA, and in some cases co-taneous anergy to skin tests was observed. Patients with SCA were less responsive to PHA than to the other intradermal skin tests. The study of these immunologic parameters in children with SCA could contribute to a better understanding of the immune system of SCA patients. The dissociation between skin tests and PHA transformation seems paradoxical, but there is evi-



Table I. Immunologic data in SCA

Case No	WBC $\times 10^9/l$	Lymphocytes		E rosettes		EAC rosettes		PHA	Skin test <sup>b</sup>
		%	$\times 10^9/l$	%	$\times 10^9/l$	%	$\times 10^9/l$		
1	9.5	10	1.0	71	0.7	38	0.4	45	4/4
2	10.5	27	2.9	72	2.0	19	0.5	33	1/2
3	9.7	31	3.0	46	1.4	10	0.3	18	0/1
4	10.1	52	5.3	65	3.4	21	1.1	56	0.4
5	13.6	30	4.1	60	2.4	23	0.9	48	0.4
6	12.1	41	5.0	70	3.5	23	1.1	52	1/4
7	10.5	25	2.6	57	1.5	25	0.7	48	3/3
8	8.8	43	3.8	68	2.6	12	0.5	42	0/3
9	11.5	27	3.1	33	1.0	16	0.5	32	0/4
10	10.8	33	3.6	89	3.2	23	0.8	40	2/3
11	15.7	18	2.8	83	2.4	24	0.7	28	3/3
12	8.7	31	2.7	70	1.9	27	0.7	28	3/4
13	4.4	28	1.2	74	0.9	10	0.1	20	1/2
14	10.6	41	4.3	65	2.8	20	0.9	38	4/4
15	4.9	16	0.8	64	0.5	27	0.2	40	4/4
16	13.3	38	5.0	36	1.8	18	0.9	26	NT <sup>c</sup>
17	8.4	20	1.7	44	0.7	38	0.6	21	NT
18	17.3	19	3.3	50	1.6	30	1.0	23	NT
19	13.7	35	4.8	56	2.7	18	0.9	48	NT
20	12.0	35	4.2	42	1.8	14	0.6	20	NT
Mean $\pm$ SD	10.8 $\pm$ 3.1	30 $\pm$ 10	3.3 $\pm$ 1.3	60 $\pm$ 15	1.9 $\pm$ 0.9	22 $\pm$ 7.8	0.7 $\pm$ 0.2	36 $\pm$ 13	
<i>Controls</i>									
Mean $\pm$ SD	6.6 $\pm$ 1.1	35 $\pm$ 7	2.4 $\pm$ 0.2	63 $\pm$ 5	1.5 $\pm$ 0.4	25 $\pm$ 6	0.6 $\pm$ 0.2	56 $\pm$ 7.6	
Range	5-10	16-46	1.2-3.7	55-75	0.7-2.3	10-33	0.3-1.1	40-72	

Lymphocyte transformation response to phytohemagglutinin.

<sup>b</sup> Number of positive skin tests/number of used antigens.

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three or four antigens, 4 of them (33%) were anergic (table I)

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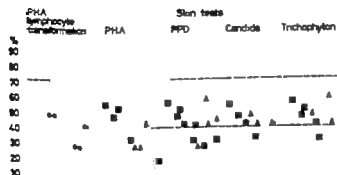


Fig. 1. Correlation between PHA lymphocyte transformation and skin test reactivity. ▲ = Positive; ■ = negative. --- = Normal range of PHA blastic transformation.

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### Discussion

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dence for the existence of subpopulations of T cells with differing properties and functions [10]. Perhaps our observation reflects independent dysfunctions of these subpopulations of T cells as it has been suggested in other pathological states [7]. Dissociation between skin tests and lymphocyte responses has been noted, with the suggestion that the *in vitro* lymphocyte reactivity may be more sensitive than the intradermal test in detecting immunity [15].

Depressed lymphocyte functions may reflect a qualitative defect in T-lymphocyte subpopulations. Other possibilities include a blocking effect of the serum or the presence of a population of suppressor T cells. Further investigations are indicated to evaluate these possibilities.

Immunoglobulin levels have been found normal in children under 4 years of age with SCA, but all three major classes were elevated in children over 4 years. In adults with SCA increased IgA levels have been reported [8]. Hyperglobulinaemia seen in SC has been related with liver dysfunction or with antigenic stimulus, although other factors might also play some role [8, 9, 17]. Depressed cell mediated immunity may be present in some SCA patients, and the hyperglobulinaemia might therefore be also an expression of compensatory B-cell over action to a wide variety of antigenic stimuli.

Defective host-defense mechanisms have been described in SCA. Among these mechanisms that may account for an increased susceptibility to infections are overloading of the reticuloendothelial system with breakdown products of haemoglobin and functional autosplenectomy abnormal neutrophil kinetics, impaired phagocytosis, defects in bacterial activity of neutrophils, slow chemotaxis, defective alternative com-

plement pathway deficiency of serum bactericidal activity deficiency of serum opsonin activity increased concentrations of free serum iron, and decreased serum transferrin levels [2, 6, 12, 14, 16, 18, 21]. We suggest that impaired cell-mediated immunity is another factor to be included among defective SCA host-defense mechanisms.

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## Ultrastructural Observations on some Unusual Lamellar Bodies Associated with Human Platelets

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**Key Words.** Lamellar bodies Platelets Ultrastructure

**Abstract.** An electron microscopic study is presented concerning unusual lamellar bodies associated with human platelets. These lamellar bodies, resembling stacks of phospholipid-like membranes, are located within vesicles, in the surface connected open canalicular system between aggregating platelets and on the free surface of platelets.

### Introduction

During the course of ultrastructural studies on human platelets from normal untreated individuals as well as from patients being treated with chlorpromazine for psychiatric disorders, we have repeatedly encountered unusual lamellar bodies. These lamellar bodies were also found during aggregation responses of platelets following the addition of a wide range of agents. We are not aware of other reports in the literature concerning similar lamellar structures associated with platelets and would like to record our observations. This present short report deals only with the unusual lamellar bodies and the pertinent data concerning the preparation of the material for electron microscopy

### Material and Methods

Blood samples, which were processed in plastic tubes, were mixed with 1/10 vol of 3.8% trisodium citrate and gently centrifuged (150 g) for 10 min. Samples of the platelet-rich plasma were fixed directly or were placed in an aggregometer (Chronolog, Havertown, Pa.) with a revolving magnet (1,000 rpm) and recordings made of aggregation responses following the addition of 5-hydroxy tryptamine (5-HT 10, 20 or 40  $\mu$ M), adenylyl 3-imido-diphosphate (AIP 100 or 300  $\mu$ M), adenosine diphosphate (ADP 0.5 or 3  $\mu$ M), epinephrine (0.5  $\mu$ M). Detailed results of these aggregation studies will be presented elsewhere.

Aliquot samples were rapidly removed at 30, 60, 90 and 120 sec time interval and placed in plastic tubes containing 2 vol of fixative composed of 3% glutaraldehyde (Ladd Chemicals) in 0.05 M Hepes buffer (Sigma) pH 7.4 with 1.23 M calcium chloride and 17% sucrose for 2.5 h at 37 C

according to *Skaer et al.* [2]. The samples were allowed to cool to room temperature during 1 h and then re-centrifuged (2,000 g) for 10 min. The fixative was poured off and replaced with fresh Hepes buffer and left overnight at 4 °C. This was followed by postfixation for 1 h in 1% osmium tetroxide in water. The samples were then dehydrated in graded concentrations of ethanol, treated with propylene oxide and embedded in Epon 812 which was polymerized at 60 °C for 24 h. 60- to 90-nm sections were cut with glass knives on an LKB Ultratome III and collected on uncoated copper grids prior to brief contrast staining with

uranyl acetate and lead citrate. Grids were examined in Jeol 100 II transmission electron microscope at 80 kV.

### Observations

Platelet-rich samples from both normal and chlorpromazine-treated donors when examined by electron microscopy showed a number of unusual lamellar bodies. These



Fig. 1. Lamellar body (arrows) within membrane-bound vesicle, possibly in this case representing the nucleus of peroxisome. 5-HT 10 V 60 sec 50,000

Fig. 2. Lamellar body in space between two platelets during aggregation. Note adjacent vesicle (V) from which the lamellar body was conceivably discharged. 5-HT 20 V 30 sec 80,000

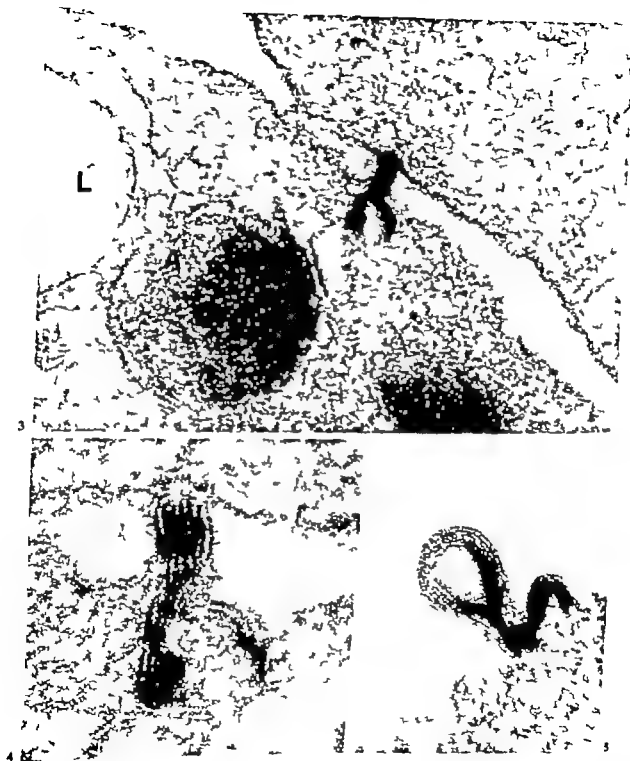


Fig. 3-5. Lamellar bodies at surface of platelets. AIP 100, Af 60 sec 3 Lamellar body bridging the gap between adjacent platelets. A = alpha-granule L = lipid droplet.  $\times 50,000$  4 Details of lamellar body seen in figure 3  $\times 150,000$  5 Lamellar body on free surface of platelet.  $\times 80,000$ .

were also found following the addition of the various agents at all the time intervals and concentration used in the experiments.

At very high magnifications ( $\times 50,000$ – $150,000$ ) the lamellar bodies were found to be composed of a series of fairly electron-dense stacked lamellae with a line-to-line separation of approximately 5–7 nm. These lamellar bodies resemble stacks of phospholipid membranes or micelles. Usually between 6 and 12 lamellae were found in each stack. In rare instances lamellar bodies were found deep inside platelets, apparently inside membrane-bound vesicles, which conceivably are peroxisomes (fig. 1). These vesicles with a lamellar nucleoid were found near peroxisome-like bodies possessing a dense peripheral linear structure and were frequently located close to  $\alpha$ -granules or lipid droplets, which sometimes possessed a more electron-dense periphery.

In several cases lamellar stacks were found within the surface-connected system of channels and some micrographs convey the impression of cisterns discharging their lamellar contents into this open canalicular system. In many cases lamellar bodies were found bridging the space between adjacent platelets during aggregation or apparently being discharged into this space (fig. 4). In the aggregating platelets typical dense bodies were present close to the platelet surface.

In the majority of cases the lamellar bodies were present close to or in conjunction with the surface membrane of the platelets. They were often found within depressions or in invaginations of the plasma membrane often close to the base of pseudopodia. The form adopted by the lamellar bodies on the surface of the platelet was often

myelin-like with an oval racetrack appearance (fig. 5) that occasionally showed a denser core, or even a 'fingerprint' or lipid multicellular pattern.

## Discussion

Despite the profuse literature on the aggregation of platelets and the fact that so much is known concerning the uptake or secretion of a wide range of chemical constituents, there are relatively few ultrastructural reports showing the processes of endocytosis or exocytosis [1, 4, 5]. At this preliminary stage we are unable to determine unequivocally the nature or significance of the lamellar bodies described in this report. In view of the fact that the lamellar structures are found in the untreated controls, this would seem to indicate that they are not specifically associated with the processes of adhesion, aggregation or secretion and are not a response to chlorpromazine treatment. Whereas it is known that the fixation procedure adopted stabilizes some platelet structures much better than other fixation methods [2, 3], it remains a distinct possibility that the lamellar structures are artifacts resulting from the mode of preparation. One further possibility to be considered is that similar lamellar structures have not been reported so far or overlooked as they entail a degree of patient searching and are only clearly seen at high magnifications.

The source of the phospholipid-like material seen in our micrographs is not known and it may be that it represents liposomes formed from some constituent in the plasma, which adheres to or reacts with specific sites of the platelet plasma membrane. This, however, does not explain the apparent



presence of lamellar structures deep inside platelets. It is possible that the lamellar structures seen in our preparations arise from more than one source. Those present deep within platelets may be derived from components of one of the organelles or inclusions such as from peroxisomes,  $\alpha$ -granules, lysosomes, lipid droplets or the serotonin-containing dense bodies, and this phospholipid like material could conceivably be extruded to the surface especially during aggregation via the open canalicular system. The more common micelle like structures seen on the platelet surface may be of an external plasma origin and may be artifacts formed during the *in vitro* preparation of the platelets for electron microscopy.

At this stage we are still unable to answer the questions raised and are continuing our studies to try and establish the origin nature and possible significance, if any to platelet morphology of these unusual lamellar structures.

### Acknowledgements

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also like to thank Dr. Blum Oppenheim for her constant help and advice during the course of the study.

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## Congenital Combined Factor VII and Factor VIII Deficiency

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**Key Words.** Factor VII Factor VIII Combined deficiency

**Abstract.** This paper describes two family members who have a combined hereditary deficiency of factors VII and VIII. The propositus, a 16-year-old male, presented with recurrent gastrointestinal bleeding. He and his mother had moderate defects of factors VII and VIII. The propositus received factor VIII cover during and after a laparotomy. The possible hereditary transmission pattern of the combined defect is discussed.

Combined hereditary deficiencies of coagulation factors are very rare disorders. In this case we describe a boy with a combined factor VII and factor VIII defect who presented with recurrent gastrointestinal bleeding which required a laparotomy.

### Case Report and Results

C.C. 16-year-old Caucasian male from Greece, presented with intermittent haematemesis, melena, stools and feeling faint for 2 months. Several other members of the family had positive bleeding history. A younger brother died at the age of 6 years due to uncontrollable post-operatively bleeding following tonsillectomy. The mother has easy bruising and bleeds excessively after tooth extraction. 2 maternal female aunts and maternal male cousins also have bleeding tendency. There was no bleeding history amongst

the paternal relatives. The parents are not consanguineous.

The propositus had had recurrent gastro-intestinal bleeding since the age of 6 years and had bled excessively after tooth extraction. He was suspected to have haemophilia A in Greece. A routine coagulation study on admission (table I) revealed combined factor VII and factor VIII defect. Liver function tests were within normal limits. There was no shortening of the prolonged prothrombin time or rise in the factor VII level after the administration of vitamin K<sub>1</sub>. Upper gastrointestinal endoscopy under cryoprecipitate cover revealed duodenal ulceration. A laparotomy was then undertaken and vagotomy and Billroth II partial gastrectomy performed. During the operation and post-operative period he received regular 1-hourly infusions with cryoprecipitate or factor VIII lyophilized concentrate (in addition factor VIII procoagulant (VHIC) levels below 50% during the operation and first 24-hour post-operative period and above 30% for the following 31 days. The half-life of the infused factor VIII was ap-

Table I. Coagulation study in propositus and parents

Test	Propositus	Mother	Father	Normal values
Ivy bleeding time, min	6	3	4%	1-5
Platelet count, $\times 10^9/l$	230	198	320	150-400
Coagulation time, min	5½	3	2½	0-6
Prothrombin time, sec	20	16	12	12
Partial thromboplastin time sec	43	40	33	2-38
Thrombin time, sec	1	12	12	12
Stypven time sec	13	13	12	12
Factor VII %	16	28	110	80-120
Factor VII antigen, %	37	90	80	70-110
Factor VIII C <sub>i</sub>	18	39	100	50-200
Factor VIII antigen	189	140	115	50-200
Factors II V IX, X, XI XII	normal	normal	normal	-
Ristocetin 1.2 mg/ml aggregation, %	70	75	80	>65

proximately 11 h. No factor VII replacement therapy was given, and factor VII activity ranged between 15 and 22%. Repeat endoscopy 33 days post-operatively showed healing of the anastomosis. Factor VIII replacement therapy was then stopped.

Coagulation studies of the parents (table I) revealed that the mother also had a combined deficiency of factor VII and factor VIII but the father had a normal screen.

## Discussion

Both the propositus, C. C., and his mother have a combined deficiency of factor VII and factor VIII. The presence of a normal bleeding time, normal factor VIII associated antigen, normal platelet aggregation to ristocetin and an effective half life of 11 h of infused factor VIII material suggest that the factor VIII defect is of a haemophilia A type. The higher levels of factor VII antigen as compared to activity are similar to the levels found in patients with isolated congenital factor VII deficiency [Denson *et al* 1972]. The propositus maintained a fac-

tor VII level above 15% without requiring any specific replacement therapy throughout the post-operative period. A factor VII level of above 10% has been reported as sufficient to assure normal haemostasis during and after major surgery [Marder and Shulman 1964].

Three other families with combined deficiency of factors VII and VIII have been satisfactorily documented [Gaston *et al*, 1961; Girolami *et al* 1976, 1977].

The first report [Gaston *et al* 1961] concerned a 22 year-old male with a factor VIII coagulant level of 1/ and a factor VII level of 55% (but no family history of a bleeding tendency or family studies).

The second family contained 6 members presenting with a combined deficiency of factor VII and VIII coagulant activity and normal or near normal levels of factor VII antigen and normal levels of factor VIII antigen [Girolami *et al* 1976]. They suggested that these patients lacked a gene on an autosomal chromosome involved in activation of factors VII and VIII.

In the third family [Girolami *et al* 1977] the mother of the propositus was a haemophilia A carrier and the father a heterozygote for factor VII deficiency. Here the combined deficiency appears to be due to the casual association of two independently segregating defects.

Our family could represent the casual association of two abnormalities appearing in both mother and son. However the factor VII and VIII levels in mother and son were similar and 2 female relatives also had significant bleeding histories. This would support an autosomal gene defect involved in factor VII and VIII activation. Unfortunately no other family members were available for investigation in Britain.

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Coagulation time, min	5 $\frac{1}{2}$	3	2 $\frac{1}{2}$	0-6
Prothrombin time, sec	20	16	12	1
Partial thromboplastin time, sec	43	40	33	32-38
Thrombin time, sec	1	12	12	12
Stypven time sec	13	13	12	12
Factor VII $\%$	16	28	110	80-120
Factor VII antigen, $\%$	37	90	80	70-110
Factor VIII C, $\%$	III	39	100	50-200
Factor VIII antigen, $\%$	189	140	115	50-200
Factors II V IX, X, XI XII	normal	normal	normal	-
Ristocetin 1.2 mg/ml aggregation, $\%$	70	75	80	>65

proximately 11 h. No factor VII replacement therapy was given, and factor VII activity ranged between 15 and 22%. Repeat endoscopy 33 days post-operatively showed healing of the anastomosis. Factor VIII replacement therapy was then stopped.

Coagulation studies of the parents (table I) revealed that the mother also had a combined deficiency of factor VII and factor VIII but the father had a normal screen.

## Discussion

Both the propositus, C. C. and his mother have a combined deficiency of factor VII and factor VIII. The presence of a normal bleeding time, normal factor VIII associated antigen, normal platelet aggregation to ristocetin and an effective half life of 11 h of infused factor VIII material suggest that the factor VIII defect is of a haemophilia A type. The higher levels of factor VII antigen as compared to activity are similar to the levels found in patients with isolated congenital factor VII deficiency [Denson *et al.* 1972]. The propositus maintained a fac-

tor VII level above 15% without requiring any specific replacement therapy throughout the post-operative period. A factor VII level of above 10% has been reported as sufficient to assure normal haemostasis during and after major surgery [Marder and Shulman 1964].

Three other families with combined deficiency of factors VII and VIII have been satisfactorily documented [Gaston *et al.* 1961, Girolami *et al.* 1976, 1977].

The first report [Gaston *et al.* 1961] concerned a 22 year-old male with a factor VIII coagulant level of 1% and a factor VII level of 55% (but no family history of a bleeding tendency or family studies).

The second family contained 6 members presenting with a combined deficiency of factor VII and VIII coagulant activity and normal or near normal levels of factor VII antigen and normal levels of factor VIII antigen [Girolami *et al.* 1976]. They suggested that these patients lacked a gene on an autosomal chromosome involved in activation of factors VII and VIII.

In the third family [Girolami *et al.*, 1977] the mother of the proband was a haemophilia A carrier and the father a heterozygote for factor VII deficiency. Here the combined deficiency appears to be due to the casual association of two independently segregating defects.

Our family could represent the casual association of two abnormalities appearing in both mother and son. However the factor VII and VIII levels in mother and son were similar and 2 female relatives also had significant bleeding histories. This would support an autosomal gene defect involved in factor VII and VIII activation. Unfortunately no other family members were available for investigation in Britain.

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## Short Communications

*Acta haemat.* 63 170-171 (1980)

### Acute Leukaemia after Exposure to a Weed Killer, 2-Methyl-4-Chlorphenoxyacetic Acid

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**Key Words.** Acute leukaemia 2 Methyl-4-chlorphenoxyacetic acid  
Occupational leukaemia Weed killers

**Abstract.** Acute leukaemia is known to develop in many cases of benzene-induced pancytopenia [1]. This is a report of the development of acute leukaemia in a patient who had apparently recovered from pancytopenia after chronic exposure to a weed killer 2 methyl-4-chlorphenoxyacetic acid.

#### Case History

The early course of our patient has been published earlier during his recovery period [4]. He was a 64-year-old farmer from North Finland. The history of his exposure to weed killers had extended for at least the 5 previous summers, and on the last occasions the preparation contained 2-methyl-4-chlorphenoxyacetic acid (MCPA). The exposure had been extensive: he had employed an old manual spreading device with a leaking container carried on his back, and the weed killer solution had moistened his clothes. On first diagnosis, his drug history for any agents known to cause cytopenia was negative for at least the last 3 years. 3 months earlier he had observed skin bruises, but he visited the local doctor only when he felt himself more severely lethargic. During this period his lowest blood values were: Hb 7.3, leucocytes 2,400 with 44% of neutrophils and platelets 37,000.

He was treated with prednisolone and methenolone, and 5 months later he had blood levels of Hb 13.5, leucocytes 7,600 with 46% of neutrophils and platelets 190,000. He was free of symptoms and was lost for further follow-up.

Unexpectedly 12 months later he appeared again after having had cough and fever for 3 weeks. The local doctor noted a mild anaemia (Hb 9.3), a moderate leucocytosis (24,000) and a thrombocytopenia (64,000), and referred him to the University Hospital. At this phase, his bone marrow smear was characteristic of myelomonocytic acute leukaemia, with Auer rods in many blast cells. In the peripheral blood, the leucocyte count rose steadily up to 91,800, with only about 5% of blast cells and about 30% of monocytes and monocytoid cell forms. The platelet count was around 20,000. The chromosome study revealed a normal karyotype, 46, XY without any Philadelphia chromosome. He did not respond to therapeutic attempts with prednisolone and died 3 weeks later of acute cerebral haemorrhage.

#### Comments

In our case the course was consistent with previously published cases of acute leukaemia after chronic exposure to ben-

zene [1] there was an extensive exposure history of several years, a pancytopenic episode with an apparently full haematological recovery and a symptom free period ending with an acute myelogenous leukaemia. The cellularity of the bone marrow has not been sufficiently predictive for subsequent development of acute leukaemia [1] and in our case the marrow was of normal cellularity during the pancytopenic period. Although the causal relationship of the exposure to benzene and the subsequent acute leukaemia is well established, leukaemia ultimately develops in only a minority of the cases with pancytopenia. As the clinical course in our case was closely similar to the events after exposure to benzene, we think that MCPA was very probably the causative agent of leukaemia.

Very recently attention has been directed to the doubling of the incidence of acute myelogenous leukaemia among older men during the last decades in Minnesota [3]. Indeed, it is in this group of people that chronic occupational and environmental exposures are most likely to result in manifest disease, and this finding is a severe challenge to find the agents liable for this development. Petroleum products containing benzene are, of course, among the first of the suspected causes [ ] Insecticides, other well-known reasons for pancytopenia, are extensively used in modern agriculture along with the weed killers. Indeed, in our previous study 7 (15%) leukaemia patients

but only 3 (6%) controls had handled weed killers or agricultural insecticides containing the benzene ring [5]. These patients along with our case report should be seen as examples where to look for agents other than benzene which could be responsible for the alarming increase of myelogenous leukaemia.

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## Short Communications

Acta haemat 63 170-171 (1980)

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## Development of Acute Myeloid Leukaemia during Lithium Treatment

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**Key Words.** Acute myeloid leukaemia Granulocytosis Lithium treatment  
Manic-depressive illness

**Abstract.** Development of acute myeloid leukaemia in 2 patients given long term lithium treatment for affective illness is reported. The 1st was a 49-year-old man who was found to have acute myelomonocytic leukaemia after about 1 year of lithium treatment. He died 2 days after admission from haemorrhagic pericarditis. The 2nd patient was a 32-year-old woman given lithium for about 12 years. Thereafter she developed acute myeloid leukaemia within a few months. A remission induction regime with daunorubicin and cytosine arabinoside was successful and she is now in a full remission state.

### Introduction

Lithium may affect granulocyte production. During lithium treatment of affective illness granulocytosis is often observed, and lithium administration has been found therapeutically effective in certain granulocytopenic states, e.g. Felty's syndrome [1] and granulocytopenia due to cancer chemotherapy [4-5]. Orr and McKernan [3] on the other hand recently reported relapse of myeloid leukaemia after 7 weeks of lithium treatment. We have observed development of acute myeloid leukaemia in 2 patients given long-term lithium treatment for affective illness.

### Case Reports

#### Case 1

A 49-year-old man was treated with lithium for recurrent endogenous depression. For periods he was also given haloperidol and clomipramine. After about 1 year of lithium treatment he developed malaise and was admitted 3 weeks later in a very poor condition with high fever, bleedings of the skin and mucosa, and congestive heart failure. Haemoglobin was 6.2 mmol/l, the serum lysosyme level was markedly elevated (4.3  $\mu$ mol/l), and the blood count showed  $50 \times 10^9$  thrombocytes per litre and  $90 \times 10^9$  myelomonocytic blasts per litre. The bone marrow was hyperplastic and dominated by myelo-monocytic blasts. 2 days after admission the patient died from haemorrhagic pericarditis.

#### Case

A 32-year-old woman was treated with lithium for bipolar manic-depressive illness. For periods

I thank professor M. Schou for valuable advice.

she was also given haloperidol and clonidine. After 12 years of lithium treatment she began feeling tired, and within a few months her haemoglobin fell from 111 to 3.5 mmol/l. The patient then became febrile, and leucocyte count revealed agranulocytosis. The bone marrow was normocellular but it was dominated by myeloid blasts, some with Auer rods. A remission induction regime with daunorubicin 45 mg/m<sup>2</sup>/day on the 1st to 3rd days (leucocyte) and cytosine arabinoside 100 mg/m<sup>2</sup>/day on the 1st to 7th days (neutrophils) was successful. The patient achieved full remission state. She is now given maintenance therapy with cytosine arabinoside and thioguanine every 5th week.

### Discussion

Lithium-induced granulocytosis seems to be due mainly to enhancement of the production of colony-stimulating activity [2]. However Orr and McKernan [3] hypothesize that lithium might also recruit granulocyte precursors from a resting to a growing state. They consider this a possible explanation of their observation of a relapse of myeloid leukaemia and suggest that lithium administration may be harmful for patients with malignancy involving granulocytic series.

Acute myeloid leukaemia is a rare disease. In Denmark about 180 new cases are recorded per year: one seventh of these in our Department. The 2 cases reported here were seen within 1 year. No previous insti-

ances have been reported of acute myeloid leukaemia developing during lithium treatment. It is an open question whether in our patients the relation is causal or coincidental.

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## Development of Acute Myeloid Leukaemia during Lithium Treatment

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#### Case 2

A 32-year-old woman was treated with lithium for bipolar manic-depressive illness. For periods

<sup>1</sup> I thank professor M. Schou for valuable advice.

as well as hemoglobins S and C, and Gd<sup>1</sup> also reflect an African gene source, but since they are subject to the selective advantage pressure of *Plasmodium vivax* [15] and falciparum malaria respectively their incidence cannot be used to determine the extent of the historic African accrual to the contemporary Sicilian genetic constitution. On current information to date, the present-day Sicilian gene pool has a small, significant contribution of African (Negroid) genes, from contacts either numerically minor or for brief time spans.

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## Letter to the Editor

Acta haemat. 63 174-175 (1980)

### 'African' Genetic Markers in Sicilians

Sandler *et al* [1] reported the presence of at least one characteristic African gene marker in the form of red cell phenotypes Jsa 3%, Fy (a-b-) 1% cDe (Rho), 3% and V (hr<sup>V</sup>), 7%, in 100 random Sicilians and in 64 family members, in whom 49 (77%) had the sickle hemoglobin gene. This has added to current knowledge of the African contribution to the gene pool of Mediterranean peoples. In a larger series, the cDe gene occurred in 1.1 and 2.8% of 707 and 800 Palermo Sicilians respectively [2, 3]. The cDe gene approaches 60% in most Negro populations of Africa and the Americas, with some 2-5% in Caucasoids of Europe (non-Mediterranean) and North America, and 5-20% occurrence in Amerindians, Mongoloids (Burmese, Japanese, Tibetans, Koreans), Lapps, Tahitians and Australian aborigines [4]. If the cDe gene is to be regarded as an African marker it must be of very considerable antiquity with early widespread dissemination to both Caucasoid and Mongoloid groups alternatively a multicentric origin should be considered, with the main focus in Africa and subsidiary origins (probably more recent) in the Near East and Far East.

There is a single report involving the occurrence in a Sicilian family of red cell glucose-6-phosphate dehydrogenase (Gd) A isoenzyme and its A- mutant [5]. Gd<sup>A</sup> and Gd<sup>A-</sup> variants are virtually entirely confined to contemporary Negroids or those with Negroid admixture (manifesting their historic origin), while Gd<sup>A</sup> and Gd<sup>B</sup> incidence reflects microenvironmental influence, e.g. malaria. In many African populations tested [6, 7], Gd<sup>A</sup> and Gd<sup>A-</sup> comprise 10-40% of this en-

zyme polymorphism, compared with over 99% for Gd<sup>B</sup> in all other non-Negroid groups.

The immunoglobulin G (IgG) polymorphism Gm, provides a haplotype series highly characteristic for particular ethnic groups. For 263 Sicilians tested [8], the recorded haplotypes were typically Caucasoid, with no evidence of Negroid types, including the uniquely specific Gm 1 5, 6, 17 haplotype [8].

Hemoglobin C, a dislocative West African variant, as well as sickle hemoglobin, has been reported in Sicilians [9]. In a uniquely different approach to the single vs. multiple mutation theory of the origin of sickle hemoglobin, restriction enzyme mapping studies of DNA structure associated with the sickle hemoglobin gene have shown heterogeneity [10, 11]. In Sicily and Cyprus, the  $\beta^S$ -globin gene is associated with the 130 kilobase fragment (as is the  $\beta^A$ -globin gene), and corresponds to that isolated from American Negroes. This suggests that the sickle hemoglobin gene in these two Mediterranean islands is of African origin. However in people from India and Saudi Arabia with the sickle hemoglobin mutation, the  $\beta^S$ -globin resides in the 76 kilobase fragment, indicating that the sickle gene in A is arose from a separate mutation. Thus, one can surmise that sickle hemoglobin arose not only in diverse ethnic groups at different geographic sites (Africa, Asia and the Mediterranean basin, but (possibly) also at different times in the past [12, 13].

Genetic markers Rho, cDe, hr<sup>V</sup> (V), Jsa and Gd<sup>A</sup> provide information regarding the degree of African admixture, best analyzed by a multivariate approach [14]. The red cell antigen Fy (a-b-),

# Effect of Aspirin and Aspirin Lysinate on Platelet Function in Smokers and Non-Smokers

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**Key Words.** Aspirin lysinate · Bleeding time · Platelet adhesivity · Platelet aggregation

**Abstract.** When administered orally both acetylsalicylic acid and acetylsalicylic acid lysinate produced an increased bleeding time, reduced platelet adhesiveness and inhibition of the second phase of ADP-induced platelet aggregation in the majority of subjects. The effects on bleeding time and adhesiveness were similar for both drugs. Platelet aggregation studies demonstrated slight differences between smokers and non-smokers, in both control samples and in response to the two drugs. It is concluded that aspirin lysinate should provide a good candidate for clinical investigation, to assess its value in the treatment of thrombotic disease.

## Introduction

There has been considerable interest in recent years in the use of drugs which inhibit platelet functions for the treatment of cardiovascular diseases associated with thromboembolism [23]. To be clinically useful, such drugs should be non-toxic, effective when taken orally and require infrequent administration [14]. Acetylsalicylic acid (aspirin) is a possible candidate for clinical trials in this area, and a number of investigations have been performed to assess its value in the therapy of thrombotic disease, with variable success [9-10].

The relative insolubility in water and gastric irritant action of acetylsalicylic acid

are limitations in the therapeutic applications of this drug [2]. However the lysine salt of acetylsalicylic acid (Aspegic; Egis, Amilly France) is soluble in water, produces very little gastric irritation in animals and man [3] and is better supported than acetylsalicylic acid by human patients requiring long-term therapy [20]. The present study of platelet functions in response to acetylsalicylic acid lysinate was carried out to assess the potential value of this drug as a therapeutic agent for thromboembolic diseases. Furthermore, due to the known association between smoking, cardiovascular disease [1] and platelet function, the study was carried out using both smokers and non-smokers.



## Book Reviews

*H. B. Williams*

**Serology Immunology and Blood Banking**  
**Functional Medical Laboratory Technology**  
**A Comprehensive Series of Manuals**  
AVI Technical Books Inc. Westport 1978  
VIII + 84 pp. US \$ 9.00  
ISBN 0-87055-766-X

This booklet has been published in a series of manuals dealing with laboratory technology. In 17 chapters serological procedures are outlined starting with the care and handling of pipettes and the preparation of serum and plasma. This is followed by several chapters which describe the serology of rheumatism and infectious mononucleosis. The syphilis serology includes VDRL and the rapid plasma reagin card test as well as the automated reagin test (AutoAnalyzer). In three main chapters basic techniques in blood banking are described: ABO and Rh typing, testing for D, cross-matching and antibody screening. The booklet is written for the practical training of medical technologists and

can also be used by teachers who are in charge of training these people. S. Seidl, Frankfurt am Main

*Stanley L. Larnberg and Robert Rothstein*  
**Histology and Cytology**  
**Functional Medical Laboratory Technology**  
**A Comprehensive Series of Manuals**  
AVI Technical Books, Inc., Westport 1978  
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This book provides a good introduction to histological techniques. The essentials of the practical work and some theoretical aspects have been outlined in an intelligible way. Therefore, this book is very useful for beginners. Those who are more experienced may miss some current techniques such as the Alcian blue method or staining of the reticulum by silver impregnation.

P. A. Gréllier

sodium salt) to give final concentrations of 2.3 or 11.7  $\mu\text{M}/\text{ml}$  plasma. The difference in transmission between PPP and PRP provided 0-100 scale of transmission. The features of the aggregation curves measured for comparative purposes are presented in figure 1 and were as follows:  $y^1$  = rate of first phase of aggregation;  $t^1$  = time in seconds to reach maximum aggregation;  $ht$  = maximum aggregation achieved expressed as a percentage of light transmission on the FRP-PPP scale;  $y^2$  = rate of first phase of disaggregation;  $t$  = time in seconds to start of disaggregation phase, and  $ht$  = degree of residual aggregation at 10 min expressed as percentage of the FRP-PPP scale.

In addition, an index of the magnitude of disaggregation, referred to in the text as 'effective disaggregation' was calculated according to the formula:

$$\frac{ht - ht^2}{ht} \times 100.$$

For samples in which ADP induced the biphasic aggregation curve, absence of disaggregation at 10 min was considered  $ht$  indicate the occurrence of an irreversible second phase. In some cases combined first and second phases were followed by a very slight degree of disaggregation. For these cases we considered that the second phase had been induced, provided effective disaggregation at 10 min was less than 5%.

Means for the groups were calculated and compared statistically using the two-tailed student 't' test or the paired 't' test. Control values obtained at -24 h and -15 min were pooled to give combined control mean. Differences were considered to be significant at  $p < 0.05$ .

## Results

Throughout the study the volunteers were in good health and no clinical effects of ASA and ASL were observed. In general the three tests revealed marked variations between individuals, but most subjects showed consistency of response to repeated tests.

### Bleeding Time and Adhesiveness (table I)

There was no significant difference in bleeding time between the mean control values of smokers and non-smokers. Both ASA and ASL produced an approximately two-fold increase in bleeding time in all subjects. This effect persisted until 24 h. Control mean adhesiveness values of smokers were similar to those of non-smokers (table I).

Table I. Bleeding time and adhesiveness in non-smokers and smokers

	ASA			ASL		
	control	+ 2 h	+ 24 h	control	+ 2 h	+ 24 h
<i>Bleeding time, sec</i>						
Non-smokers	100 $\pm$ 28	173 $\pm$ 63**	191 $\pm$ 85	90 $\pm$ 23	214 $\pm$ 99*	169 $\pm$ 108
Smokers	85 $\pm$ 18	145 $\pm$ 60*	111 $\pm$ 16*	89 $\pm$ 28	186 $\pm$ 106	236 $\pm$ 190*
<i>Adhesiveness, %</i>						
Non-smokers	78.9 $\pm$ 6.6	25.8 $\pm$ 6.9	22.3 $\pm$ 4.3*	30.5 $\pm$ 6.6	25.2 $\pm$ 6.4	27.8 $\pm$ 6.8
Smokers	30.2 $\pm$ 8.5	22.1 $\pm$ 6.8**	26.7 $\pm$ 9.6	23.7 $\pm$ 5.7	22.2 $\pm$ 5.2	24.4 $\pm$ 7.4

Values are expressed as group means  $\pm$  1 SD. Statistically different from pre-treatment values: \*  $p < 0.05$   $p < 0.01$



gregation occurred in all subjects at 2 and 24 h following ASA and ASL administration. The time to the start of disaggregation ( $t$ ) was shorter with ASL than with ASA at 24 h ( $p < 0.02$ ). ASL also gave rise to more rapid disaggregation rate values ( $y^*$ ) at 24 h ( $p < 0.02$ ) than ASA. The effective disaggregation at 10 min was clearly greater for ASL at 2 h ( $p < 0.02$ ) and at 24 h ( $p < 0.001$ ) than for ASA.

**11.7  $\mu$ M ADP** For subjects in which disaggregation occurred, the disaggregation rate ( $y^*$ ) was similar with both drugs.  $t$  values were significantly shorter with ASL than with ASA at 24 h ( $p < 0.005$ ). Effective disaggregation at 10 min was greater following administration of ASL than ASA, but this was statistically confirmed for the 24-hour test only ( $p < 0.01$ ).

### Smokers. Aggregation and Disaggregation Phases (table III-IV)

In smokers, the effects of ASA were similar to those of ASL. With 2.3  $\mu$ M ADP both drugs produced an inhibition of the second phase in the majority of cases at both 2 and 24 h. With 11.7  $\mu$ M ADP induction of the second phase was present in more than 50% of the subjects at 2 and 24 h with either drug. There was a similar decrease in  $t$  and  $ht$  values following ASA and ASL treatment. Mean  $t$ ,  $y^*$  values and effective disaggregation values at 10 min were also comparable with both drugs.

### Comparison of Non-Smokers with Smokers (table II-IV)

In most cases the mean value for the rate of aggregation ( $y^*$ ) was less in smokers than

Table III. Aggregation phase

Parameter measured	ASA			ASL		
	control	+ 2 h	+ 24 h	control	+ 2 h	+ 24 h
<i>Non-smokers, 2.3 <math>\mu</math>M ADP</i>						
$y^*$	85 $\pm$ 33	91 $\pm$ 41	87 $\pm$ 40	92 $\pm$ 21	94 $\pm$ 26	99 $\pm$ 28
$t$	397 $\pm$ 134	122 $\pm$ 42**	139 $\pm$ 59**	247 $\pm$ 117	86 $\pm$ 21**	88 $\pm$ 13*
$ht$	81 $\pm$ 10	63 $\pm$ 9*	63 $\pm$ 12**	80 $\pm$ 12	63 $\pm$ 13	66 $\pm$ 10**
<i>Non-smokers, 11.7 <math>\mu</math>M ADP</i>						
$y^*$	70 $\pm$ 28	79 $\pm$ 31	73 $\pm$ 27	106 $\pm$ 31	103 $\pm$ 33	104 $\pm$ 31
$t$	153 $\pm$ 93	246 $\pm$ 71**	284 $\pm$ 83	238 $\pm$ 67	158 $\pm$ 34*	167 $\pm$ 23
$ht$	87 $\pm$ 9	84 $\pm$ 10	81 $\pm$ 8*	88 $\pm$ 9	82 $\pm$ 11	84 $\pm$ 8
<i>Smokers, 2.3 <math>\mu</math>M ADP</i>						
$y^*$	68 $\pm$ 26	80 $\pm$ 32	68 $\pm$ 25	78 $\pm$ 25	84 $\pm$ 22	69 $\pm$ 23
$t$	304 $\pm$ 170	98 $\pm$ 44	123 $\pm$ 51**	293 $\pm$ 127	212 $\pm$ 34**	145 $\pm$ 64**
$ht$	69 $\pm$ 19	57 $\pm$ 14**	60 $\pm$ 9*	78 $\pm$ 20	59 $\pm$ 13**	57 $\pm$ 16**
<i>Smokers, 11.7 <math>\mu</math>M ADP</i>						
$y^*$	69 $\pm$ 23	67 $\pm$ 24	70 $\pm$ 29	70 $\pm$ 24	81 $\pm$ 17	75 $\pm$ 21
$t$	314 $\pm$ 70	282 $\pm$ 67	257 $\pm$ 41**	344 $\pm$ 51	268 $\pm$ 73	250 $\pm$ 59*
$ht$	86 $\pm$ 9	78 $\pm$ 8	81 $\pm$ 5	80 $\pm$ 11	81 $\pm$ 9	79 $\pm$ 8

Statistically different from pre-treatment values at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$

Both ASA and ASL produced a moderate decrease in adhesiveness at 2 h whilst at 24 h all groups except ASA treated non-smokers had returned towards their control values.

### Aggregation

Platelet counts for PRP before adjustment with PPP showed no significant difference between treatment groups or between smokers and non-smokers. After adjustment the counts were close to the desired value of  $250 \times 10^3/\text{mm}^3$  with a range of group means from 233 to  $270 \times 10^3/\text{mm}^3$ .

Marked differences in the shape of aggregation curves were observed between subjects but, for individual subjects, repeated tests gave surprisingly consistent results. Results of analyses of the aggregation curves are presented in tables III and IV.

### Comparison of ASL with ASA

#### Non-Smokers Aggregation Phase (table III)

**2.3  $\mu\text{M}$  ADP** Prior to drug treatment, 2.3  $\mu\text{M}$  ADP produced the classic biphasic aggregation curve in the majority of cases (table II). Both ASA and ASL inhibited the second phase at 2 h in all subjects. At 24 h

this effect persisted in all ASL subjects and in 90% of ASA-treated subjects. There was no apparent effect of ASA or ASL on the rate of the first phase of aggregation ( $y'$ ). The maximum degree of aggregation ( $\text{ht}$ ) was reduced to a similar degree with both drugs at 2 and 24 h. All mean  $t'$  values were less during the ASL than during the ASA study but both treatments produced an evident reduction of  $t'$  values at 2 and 24 h.

**11.7  $\mu\text{M}$  ADP** The majority of control samples exhibited an irreversible second phase of aggregation (table II). Following ASL treatment, inhibition of the second phase was observed in all subjects at 2 h and in 90% of them at 24 h. In contrast, with ASA, the majority of subjects showed induction of the second phase at these two intervals. Neither ASA nor ASL altered the rate ( $y'$ ) or the degree ( $\text{ht}$ ) of the first phase of aggregation. Mean  $t'$  values were similarly reduced with both drugs, but less than with 2.3  $\mu\text{M}$  ADP.

#### Non-Smokers. Disaggregation Phase (table IV)

**2.3  $\mu\text{M}$  ADP** In the majority of control samples 2.3  $\mu\text{M}$  produced induction of the second phase (table II). In contrast, disag-

Table II. Percentage of subjects showing induction of the second aggregation phase

	ASA			ASL		
	control	+ 2 h	+ 24 h	control	+ 2 h	+ 24 h
<i>Non-smokers</i>						
2.3 $\mu\text{M}$ ADP	89	0	10	79	0	0
11.7 $\mu\text{M}$ ADP	94	70	80	95	0	0
<i>Smokers</i>						
2.3 $\mu\text{M}$	65	0	10	74	0	0
11.7 $\mu\text{M}$ ADP	90	40	60	100	60	70

of aspirin on adhesiveness using 3 g/day for 2½ days. The slight reduction observed with ASA and ASL in the present study together with the large individual variations, suggests the need for large group sizes for detecting this slight, but potentially important action of these drugs. Increased platelet adhesiveness has been reported as an acute effect of smoking [11]. In the present study no differences in adhesiveness or bleeding time were found between smokers and non-smokers but no attempt was made to distinguish between acute and chronic effects of smoking.

A wide range of different criteria has been applied to the assessment of platelet aggregation curves induced *in vitro* by ADP. These criteria have included the presence or absence of the second phase [16], concentration of ADP required to induce the second phase [5], rate of aggregation and time to disaggregation [18], maximum aggregation expressed as a percentage of the PPP PRP scale [23] or as 'units' on this scale [8]. Due to technical constraints we confined our study to two concentrations of ADP. We selected the PRP PPP scale of light transmission as this has been used by other workers [11, 23]. However there was some variation in the size of this scale on the recorder even between repeated tests in the same subject. This suggests that this scale might not be ideal and we would therefore recommend that true light transmission values also be recorded.

Results of the present study have demonstrated that, overall, the effects of ASL on platelet aggregation were similar to those of ASA. However in non-smokers ASL inhibited the second phase of aggregation in a clearly greater proportion of subjects than ASA. This difference between the two drugs

was evident with 11.7  $\mu$ M ADP at both 2 and 24 h, suggesting that this effect was not due to the slower absorption of ASA. In smokers, however, the percentage of subjects in whom the second phase was inhibited was similar with ASA and ASL, suggesting that smoking can affect the pattern of aggregation curves and that the choice of subjects for such studies should be defined with respect to smoking habits.

Disaggregation of platelets *in vivo* may be an important feature of haemostasis, permitting platelets not intimately incorporated into a plug to return to the circulation [4]. In an attempt to better define the magnitude of disaggregation we proposed the use of effective disaggregation which indicates the degree of disaggregation expressed as a percentage of the maximum aggregation achieved. Whether 'effective disaggregation' reflects at all the situation *in vivo* has yet to be established, but this represents a potentially useful aspect of *in vitro* aggregation assessment. We found that effective disaggregation could be readily measured without extensive analysis of curves. In non-smokers we found that ASL induced a greater effective disaggregation than ASA with either 2.3 or 11.7  $\mu$ M ADP. Furthermore, the disaggregation phase was more rapid in onset and rate for ASL and ASA in these subjects. Thus, we consider that ASL is at least as effective as ASA as an inhibitor of platelet function. This, in addition to its clinical advantages [2, 20] suggests that ASL might prove to be a good candidate drug for studies in thrombotic disease.

#### Acknowledgements

The authors wish to express their gratitude to Miss H. Schmidt and Miss H. Kilian for their val-

Table IV Disaggregation phase

Parameter measured	ASA			ASL		
	control	+ 2 h	+ 24 h	control	+ 2 h	+ 24 h
<i>Non-smokers, 2.3 <math>\mu</math>M ADP</i>						
(+) $t^2$	>540	175 $\pm$ 77	212 $\pm$ 94	>470	118 $\pm$ 35	119 $\pm$ 37*
(+) $y^2$	<4	30 $\pm$ 45	21 $\pm$ 18	<5	44 $\pm$ 12	40 $\pm$ 13*
$[(ht^1 - ht^2)/ht^1] \times 100$	4.4 $\pm$ 12.2	32.7 $\pm$ 26.7	23.2 $\pm$ 19.8	6.0 $\pm$ 10.1	63.3 $\pm$ 11.3**	59.9 $\pm$ 14.6**
<i>Non-smokers 11.7 <math>\mu</math>M ADP</i>						
(+) $t^2$	>580	334 $\pm$ 126	368 $\pm$ 71	>550	275 $\pm$ 95	260 $\pm$ 42*
(+) $y^2$	<1	14 $\pm$ 5	6 $\pm$ 4	<1	14 $\pm$ 5	10 $\pm$ 6
$[(ht^1 - ht^2)/ht^1] \times 100$	0.4 $\pm$ 1.5	8.2 $\pm$ 15.7	4.6 $\pm$ 3.2	0.9 $\pm$ 1.6	18.2 $\pm$ 10	10.2 $\pm$ 4.8*
<i>Smokers, 2.3 <math>\mu</math>M ADP</i>						
(+) $t^2$	>400	124 $\pm$ 50	185 $\pm$ 98	>470	146 $\pm$ 54	193 $\pm$ 95
(+) $y^2$	<12	42 $\pm$ 36	26 $\pm$ 24	<13	55 $\pm$ 43	34 $\pm$ 30
$[(ht^1 - ht^2)/ht^1] \times 100$	11.7 $\pm$ 20.9	46.5 $\pm$ 19.3	28.8 $\pm$ 26.1	8.3 $\pm$ 17.3	35.4 $\pm$ 23.8	27.5 $\pm$ 23.6
<i>Smokers 11.7 <math>\mu</math>M ADP</i>						
(+) $t^2$	>560	322 $\pm$ 79	331 $\pm$ 97	>600	355 $\pm$ 103	290 $\pm$ 15
(+) $y^2$	<2	9 $\pm$ 6	8 $\pm$ 5	0	7 $\pm$ 6	8 $\pm$ 5
$[(ht^1 - ht^2)/ht^1] \times 100$	1.2 $\pm$ 3.0	7.4 $\pm$ 8.0	4.3 $\pm$ 5.8	0.6 $\pm$ 1.1	4.7 $\pm$ 5.8	3.9 $\pm$ 6.0

(+) = Values calculated from those subjects showing clear disaggregation phase. Statistically different from ASA values at  $p < 0.05$   $p < 0.01$   $p < 0.001$

in non-smokers for both 2.3 and 11.7  $\mu$ M of ADP. Using pooled pre or post treatment values for  $y^2$  this difference was statistically significant ( $p < 0.02$ ), indicating a slower aggregation in smokers. With 2.3  $\mu$ M ADP the maximum degree of aggregation ( $ht^1$ ) was less in smokers than in non-smokers for both control and post-treatment values, but these differences were not statistically significant. The effective disaggregation at 10 min following ASL treatment was less in smokers than in non-smokers, whilst with ASA there was no such difference.

## Discussion

The aim of this study was to compare the effects on platelet function of ASL and

ASA, in order to assess the potential value of ASL in the treatment of thrombotic disease. It is known that ASA prolongs bleeding time in man, and this test is considered to give an indication of the state of *in vivo* platelet functions [13]. In our study ASA and ASL produced a similar increase in bleeding time, which was consistent with the results obtained with ASA by Quick [17]. The two drugs thus have an equivalent action on bleeding time and therefore possibly on platelet function.

Concerning adhesiveness, our results are consistent with those of Stuart [19] who reported a very slight decrease in platelet adhesiveness to glass beads after 1 day using repeated daily doses of 300 mg aspirin. Wets [21] failed to demonstrate any effect

of aspirin on adhesiveness using 3 g/day for 2/ days. The slight reduction observed with ASA and ASL in the present study together with the large individual variations, suggests the need for large group sizes for detecting this slight, but potentially important action of these drugs. Increased platelet adhesiveness has been reported as an acute effect of smoking [11]. In the present study no differences in adhesiveness or bleeding time were found between smokers and non-smokers but no attempt was made to distinguish between acute and chronic effects of smoking.

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## Thrombocytopenia in Graves' Disease: Effect of $T_3$ on Platelet Kinetics

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**Key Words.** Graves disease Platelet kinetics Triiodothyronine

**Abstract.** A study was carried out in which the platelet count was decreased in approximately half the patients with hyperthyroidism and gradually increased with treatment. Platelet disappearance curves were curvilinear and the platelet survival was shortened in the hyperthyroid state. Patients maintained in a euthyroid state for 3 months or less continued to have a shortened platelet survival. The survival returned to normal after 6 months or more of euthyroid status. In order to clarify the cause of the decreased platelet count in the patients, animal experiments were performed.  $T_3$ -injected rats had decreased platelet counts and shortened platelet survival. When platelets obtained from  $T_3$ -injected rats were transfused to a control group of untreated rats, the platelet survivals were normal. When platelets obtained from the control group of rats were transfused to  $T_3$ -injected rats, the platelet survivals were shortened. Disappearance of heat-damaged RBC from the circulation was also accelerated in  $T_3$ -injected rats. This suggests that thrombocytopenia in Graves' disease is caused by an increased sequestration potency of the reticuloendothelial phagocyte system stimulated by thyroid hormone.

Thrombocytopenia occurs frequently in Graves' disease [1]. Several reports have been published on the close relationship between the platelet count and the thyroid function in Graves disease [2, 3] where the platelet count is decreased in the hyperthyroid state and gradually increases as thyroid function returns to normal. *Lamberg et al* [2] reported a shortened platelet life span in Graves disease. *Girsh and Myerson* [4] suggested that hypersplenism might be the

possible factor of thrombocytopenia in this disease, since splenomegaly is frequently associated with hyperthyroidism. The mechanism of thrombocytopenia in Graves disease is, however still a matter of speculation.

In order to analyze the mechanism of the decreased platelet count in Graves disease, the effect of thyroid hormone on the platelet count and the platelet life span was studied both in patients with Graves disease and  $T_3$ -injected experimental animals.

uable technical assistance, and to Dr C. R. E. Coggin for helpful discussions.

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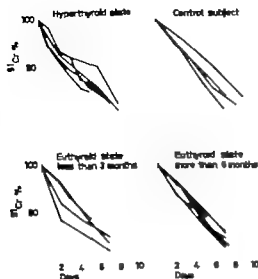


Fig. 2. Platelet survival curves in patients with Graves disease.

Half-disappearance time, days  
1 2 3 4 5 6

Control subject  
Graves disease  
Hyperthyroid state  
Euthyroid state  
Less than 3 months  
More than 6 months



Fig. 3. Changes in platelet half-disappearance time in Graves' disease.

Patients had curvilinear platelet disappearance curves (fig. 2) and significantly shortened survival of autologous platelet ( $T = 3.3 \pm 0.8$  days) as compared to control subjects ( $4.6 \pm 0.8$  days) ( $p < 0.05$ ) (fig. 3). There was no significant correlation howev-

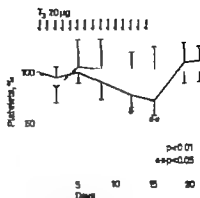


Fig. 4. Time course of changes in platelet count after triiodothyronine injection in rats. ----- = Control rat; — =  $T_4$ -injected rat.

er between platelet survival and  $T$  or  $T_4$  level. Patients maintained in a euthyroid state for 3 months or less still had a shortened platelet survival ( $3.5 \pm 1.1$  days) ( $p < 0.05$ ), similar to hyperthyroid patients. Patients who were euthyroid for 6 months or more had linear platelet survival curves and normal platelet life spans ( $4.3 \pm 0.4$  days).

Animal experiments were performed to confirm these clinical findings. Hyperthyroid rats were produced by intraperitoneal injection of triiodothyronine. A decrease in the platelet count appeared on the 8th day (fig. 4) and was marked by day 12 ( $p < 0.01$ ) and 15 ( $p < 0.005$ ). The platelet count rapidly returned to normal after the withdrawal of  $T_4$ .

Platelet kinetic studies performed on the 8th day of  $T$  injection showed a curvilinear platelet disappearance curve and shortened platelet survivals ( $T = 28.6 \pm 11.6$  h) as compared with control rats ( $42.9 \pm 2.5$  h) (fig. 5). To investigate whether the effect of  $T$  on platelet survival resulted from the

## Materials and Methods

Platelet counts in 214 patients with Graves disease were examined. The diagnosis of Graves disease was based on clinical evaluation and the results of a number of laboratory tests including serum  $T_3$ ,  $T_4$  and cholesterol levels. Untreated patients, patients receiving antithyroid drugs or  $^{131}\text{I}$ -radiation but still in a hyperthyroid state, and patients who had returned to a euthyroid state were included in the study. Platelet kinetic studies were performed in 16 patients. These patients were divided into three groups. The first group consisted of 5 hyperthyroid patients, treated and untreated. Mean  $T_3$  and  $T_4$  levels in this group were  $414 \pm 131 \text{ ng/dl}$  (normal  $159 \pm 20 \text{ ng/dl}$ ) and  $18.9 \pm 3.5 \mu\text{g/dl}$  (normal  $8.1 \pm 1.3 \mu\text{g/dl}$ ), respectively. The second group consisted of 4 treated patients who were in the euthyroid status for 3 months or less and the third group consisted of 7 patients maintained in the euthyroid status for 6 months or more. Mean  $T_3$  and  $T_4$  levels in these euthyroid patients were  $177 \pm 12 \text{ ng/dl}$  and  $9.0 \pm 1.5 \mu\text{g/dl}$ , respectively.

Platelet counting was carried out according to the method of Brecker and Cronkite [5]. A platelet kinetic study was performed with autologous platelets, labeled *in vitro* with  $^{51}\text{Cr}$  according to the method of Abrahamson [6]. Platelet kinetic studies in rats were done according to the method of Aster [7].

The animals used in these experiments were Wistar strain male rats weighing 180–220 g. Each group consisted of 6–10 rats. In order to produce hyperthyroid rats, 20  $\mu\text{g}$  of 3,3',5-triiodo-L-thyronine (Nakarai Chemicals, Kyoto, Japan) in physiologic saline adjusted to pH 9.2 by the addition of NaOH was given to each rat intraperitoneally every day. Gain in body weight of  $T_3$ -injected rats was  $1.4 \pm 1.0 \text{ g/day}$  and of saline-injected rats was  $4.8 \pm 1.4 \text{ g/day}$  ( $p < 0.001$ ). After  $T_3$  injection was continued for 13 days, serum  $T_3$  level was examined. 1.5 h after the last  $T_3$  injection,  $T_3$  level was  $899 \pm 182 \text{ ng/dl}$ , and 24 h later  $T_3$  level was  $633 \pm 145 \text{ ng/dl}$ .  $T_4$  level was  $76 \pm 32 \text{ ng/dl}$  in control rats.

The clearance rate of heat-damaged RBC from circulation was measured according to the method of Kaplan and Jandl [8]. The labeled RBC was of Kaplan and Jandl [8]. The labeled RBC was heated for 40 min at  $45^\circ\text{C}$ , thereafter 0.5 ml of  $^{51}\text{Cr}$ -labeled RBC suspension was transfused into

a rat via a tail vein, 20  $\mu\text{l}$  of the blood sample were obtained from a tail vein at 1, 15 and 24 h following injection. The statistical analyses were made using Student's *t* test and calculated on differences between matched pairs.

## Results

The platelet counts of 214 patients with Graves disease are shown in figure 1. It was not uncommon for the platelet count to be between 10 and  $15 \times 10^4/\mu\text{l}$  in untreated patients, being of normal value in approximately half the patients. The platelet count in hyperthyroid patients began to increase with treatment with antithyroid drug or  $^{131}\text{I}$ -radiation and had returned to a normal level after hyperthyroidism had been well controlled. There was no statistical correlation between platelet count and  $T_3$  or  $T_4$  level.

Platelet kinetic studies in Graves disease gave the following results. Hyperthyroid pa-

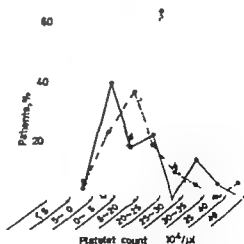


Fig. 1. Platelet count in 214 patients with Graves' disease. ●—●—● = Before treatment; ●—●—● = after treatment, hyperthyroid state; ○—○—○ = after treatment, euthyroid state.

autoimmunity does not play a main role in the shortening of platelet survival in Graves disease.

(2) Thyroid hormone - Platelets might be damaged by thyroid hormone in their survival. However patients maintained in a euthyroid state for 3 months or less had still shortened platelet survivals. Moreover in cross-transfusion experiments, the platelets obtained from  $T_4$ -injected rats had normal survivals in rats given T from the day of transfusion. It was the adequate level in the initial 3 days. These findings suggest that platelets are not directly damaged with thyroid hormone.

(3) RES system - Spleen and/or reticuloendothelial phagocyte system may be activated by thyroid hormone. Hyperplasia of these organs is commonly found in Graves disease [10]. The spleen was considered as a main sequestration site of platelets [11]. In our data, the platelet count gradually was decreased with T injection and the significant shortening of platelet survival was observed after 8 days of injection. We considered that the reticuloendothelial phagocyte system in rats might be activated with  $T_4$ . Various methods have been reported to measure phagocytic activity of reticuloendothelial organs. Accelerated disappearance of heat-damaged RBC from the circulation was revealed in hyperthyroid rats. *Frimmer* [12] also has reported that reticuloendothelial clearance for macromolecules was accelerated in hyperthyroid rats.

Patients maintained in a euthyroid state for 3 months or less still had a shortened platelet survival in our study. This indicates that clearance rate of phagocytic organs does not concomitantly return to normal with T level. The rate gradually returns to normal after serum T level is normalized.

Therefore there was no correlation between platelet count or platelet survival and  $T_4$  or T level.

We consider that the mechanism of the shortened platelet survival resulting in thrombocytopenia in Graves disease is an increased sequestration activity by the reticuloendothelial phagocyte system stimulated with thyroid hormone.

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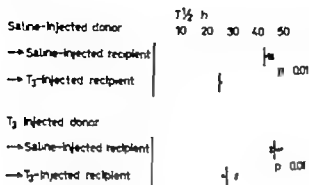


Fig. 5. Platelet half-disappearance time in cross-transfused rats.

change of the platelet itself or other factors, cross-transfusion of platelets was performed. When platelets obtained from  $T_3$  injected rats were transfused to saline-injected rats, platelet survival curves were linear and half disappearance times were normal ( $471 \pm 44$  h). On the contrary when platelets obtained from saline-injected rats were transfused to hyperthyroid rats, survival times were shortened ( $248 \pm 116$  h).

When platelets obtained from rats receiving  $T_3$  for 8 days prior to the measurement were transfused to rats receiving  $T_3$  on the day of the survival measurement, platelet survival was normal. Platelet survival on the 3rd day after cessation of 15 days of  $T_3$  injection was measured in order to study when it returned to normal. These rats had slightly shortened platelet survival but the difference was not significant. On the 10th day platelet survival returned to normal.

In order to measure the sequestration activity of the reticuloendothelial phagocytosis system (RES), clearance rates of heat-damaged RBC in  $T_3$ -injected rats were examined. RBC  $^{51}\text{Cr}$  activities (percent injected) in saline-treated rats at 15 and 24 h were  $54.6 \pm 5.6$  and  $43.2 \pm 8.0$  while in  $T_3$  injected rats they were  $37.3 \pm 7.2$  and

$248 \pm 6.2$ , respectively. The difference was marked at both hours ( $p < 0.001$ ,  $p < 0.001$ ).

## Discussion

Thrombocytopenia is a common feature of Graves disease. *Blstrom* [1] reported that severe hyperthyroid patients generally had a lower platelet count than moderate hyperthyroid patients. The results of our survey of platelet counts in patients with Graves disease clearly indicated that a decreased number of platelets was seen in hyperthyroid state. It was also commonly found that the platelet count was decreased in accordance with the degree of hyperthyroid state in ITP patients associated with Graves disease and began to increase as these patients had normal thyroid function tests [2, 3]. These findings suggest that there is a close relationship between the platelet count and thyroid function.

The mechanism of the shortening of platelet life span is suspected to be as follows:

(1) Autoimmune mechanism - Graves disease has been considered as an autoimmune disorder since long-acting thyroid stimulators were demonstrated [9]. Circulating immunocomplex was found in these patients and the platelet might be damaged by such a substance. However platelet antibody or antibody-like substance has not been reported in Graves disease. In our data, however patients who were euthyroid for 6 months or more had a normal platelet life span while the etiologic factor of Graves disease could not be eliminated. According to our unpublished data, patients with Hashimoto thyroiditis, an example of typical autoimmune disease, had normal platelet survivals. These findings suggest that

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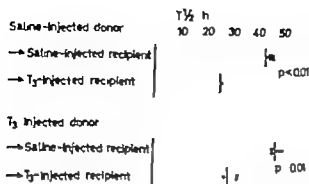


Fig. 5 Platelet half-disappearance time in cross-transfused rats.

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## The Relationship between the Blood and Vein Wall Fibrinolytic Activities in Response to Surgical Trauma

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**Key Words.** Fibrinolytic activity Surgical trauma Vein

**Abstract.** The fibrinolytic response to surgical trauma was studied longitudinally in 7 pigs by measuring the fibrinolytic activity in the blood and vein wall simultaneously. The results indicated a marked suppression in fibrinolytic activity in the blood which was closely correlated with the activity in the vein wall. This correlation lends support to the hypothesis that the venous endothelium is the source of spontaneous fibrinolytic activity in the blood.

### Introduction

The relationship between trauma and the fibrinolytic activity (FA) in the blood of man is well recognized. Within the first hour of injury or operative trauma, there is an accelerated fibrinolytic response [7 9 10] followed by a period of marked suppression [7] known as the fibrinolytic shutdown. *Fearnley* has suggested that the venous endothelium is the source of fibrinolytic activity (FA) in the blood [5 6]. In studies of human subjects, *Browne et al* [3] obtained results which are suggestive, but not statistically significant, of a correlation between blood and vein wall FA. Therefore, we studied the FA in the blood and the vein wall during the period of marked suppression using an animal which has similar fibrinolytic properties as man [2].

### Method

7 pigs each weighing 30–35 kg were used in this study. Each animal was anesthetized using oxygen, nitrous oxide, halothane and endotracheal intubation. In 2 animals autogenous grafting of the femoral veins was performed and in 5 animals abdominal skin flaps were raised and resutured. The operation time was 3 and 1½ h, respectively. The two operative procedures were similar in terms of tissue injury to the animals. From the central vein at the base of the neck, blood was withdrawn with needles before, during and after the operation on alternate days for 9 days. Within 30 min of taking the blood, which was oxalated and kept in melting ice, euglobulin clot lysis time (ECLT) was performed using the method described by *Buckell* [4]. The FA in the blood was expressed as the reciprocal of ECLT in hours multiplied by factor of 1,000 (normal range = 71–333 ECLT units; mean  $\pm$  SEM =  $147 \pm 18.5$  = 13).

Segments of the saphenous vein were biopsied from the hind leg of the pig at the time coinciding

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Table II The results of the fibrinolytic activity from 7 animals obtained before, during and after surgery

Fig No.	Source of fibrinolytic activity	Time after surgery							
		0 h	1% h	3 h	day 1	day 3	day 5	day 7	day 9
1	blood vein	133 5.8	- -	89 0.75	47 1.75	90 4.29	92 4.5	134 8.41	124 9.75
2	blood vein	125 16.92	- -	57 0.19	18 1.18	80 9.36	53 4.06	100 5.74	- -
3	blood vein	111 12.78	88 4.65		68.4 3.48	34.3 7.59	41 -	51.9 -	88.9 13.59
4	blood vein	174 19	148 15.62		120 5.56	80 0.07	91 9.77	43 -	73 31.2
5	blood vein	111 7.89	52 5.16		29.5 3.53	4.5 1.17	70 15	40.5 7.44	77.5 9.83
6	blood vein	118 -	104 10.43		- -	48.5 7.35	105 14.49	111 9.67	- -
7	blood vein	111 15.96	80 10.53		120 10.99	80.5 1.11	103.5 9.8	78 -	
Mean ± SD	blood vein	119 ± 32 13 ± 5	94.4 ± 35 9.2 ± 4	73 ± 22 0.4 ± 4	66.6 ± 45 4.4 ± 3	62.5 ± 26 4.4 ± 3	79.6 ± 24 9.6 ± 4	79.8 ± 36 7.8 ± 1	90.8 ± 23 16 ± 10
p	blood vein		p = 0.23 p = 0.23		p = 0.003 p = 0.007	p = 0.003 p = 0.005	p = 0.02 p = 0.25	p = 0.04 p = 0.09	p = 0.1 p = 0.5

Blood activity is measured by the reciprocal of ECLT 1,000. The vein activity is measured by the ratio of the area of lysis to the area of vein. p values are calculated with the fibrinolytic activity at zero hour as control.

becomes less strong when points up to the fifth day ( $r = 0.676$ , d.f. = 30  $p < 0.001$ ) the seventh day ( $r = 0.624$  d.f. = 34  $p < 0.001$ ) and the ninth day ( $r = 0.597$  d.f. = 38,  $p < 0.001$ ) are included in the analysis.

### Discussion

There is abundant evidence to show that surgical trauma will initiate a process lead-

ing to a fibrinolytic shutdown during the first week after surgery. Our study suggests that during the period of fibrinolytic suppression there is a close correlation between the FA in the blood and the vein wall. We did not find a rise in the FA before and during surgery as observed by *Marrsfield* [10] and *MacFarlane* [9]. The preoperative rise in FA is believed to be due to anxiety [10] which can be eliminated in an animal model. At least 50% of the patients studied by *MacFarlane* did not show an increase in FA

with the sampling of blood. Each specimen of vein, 5-8 mm long, was removed from the distal part of the vein between two tributaries, so that blood flow in the proximal part of the vein was maintained and multiple biopsies could be obtained from the same vein (fig. 1). The specimen of vein was immediately frozen in isopentane with carbon dioxide ice and stored at  $-20^{\circ}\text{C}$  until required. The FA of the vein was measured quantitatively using a method based on Todd's original autograph technique [11]. The details of our modification were reported recently [12]. Briefly eight frozen sections from the vein sample were each placed on a round coverslip. A thin film of topical thrombin was left on the coverslip before 0.05 ml of a 1% fibrinogen solution (containing 0.03 AC unit of plasminogen) was spread over the whole coverslip. Therefore, a fixed volume of solution was distributed over a constant area resulting in a uniform layer of fibrin over the section. After 1 h of incubation at  $37^{\circ}\text{C}$  the sections were formalized before being stained. Using a counting grid mounted onto the eyepiece of the microscope, the area of lysis was measured. The area of the vein section was measured similarly by the product of the length of the intima and the mean thickness of the vein wall. The ratio termed Fibrinolytic Index, is formulated below:

$$\text{Fibrinolytic index} = \frac{\text{Area of lysis}}{\text{Area of vein wall}}$$

The mean Fibrinolytic Index from eight separate sections of each vein represented the FA for that vein (normal range = 8-22.6, mean  $\pm$  SEM =  $13.3 \pm 0.9$ ,  $n = 22$ ).

## Results

Table I shows the FA in the blood and the vein in each animal before, during and after surgery. There was no difference in the fibrinolytic response of the animals to the two types of operative procedures. In each animal studied, there was a drop in the FA in the blood and the vein at the end of surgery. The maximum fibrinolytic suppression occurred on the first and third postoperative days. This was followed by recovery on the fifth and the seventh days. Using the Student's *t* test, the postoperative fibrinolytic measurements from each set time were compared with the preoperative levels. Although there was a fall the FA at the end of surgery the difference was not great enough to be statistically significant. On the first and third days, the activities in the blood and that in the vein wall were significantly lowered. The simultaneous measurement of the FA in the blood and the vein from each animal at the specified time after surgery constitute a pair of readings which is represented by a point in figure 2. When all the points from up to the third postoperative day are analyzed, a linear relationship is shown with good correlation ( $r = 0.728$ , d.f. = 24,  $p < 0.001$ ). This correlation

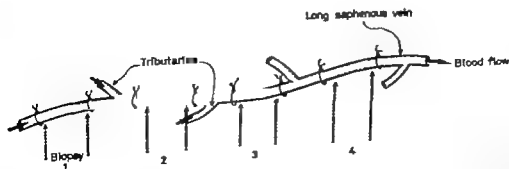


Fig. 1. Method of vein biopsy. The first biopsy is done distally between two tributaries. Subse-

quent biopsies, as indicated, do not interrupt normal blood flow in the proximal part of the vein.

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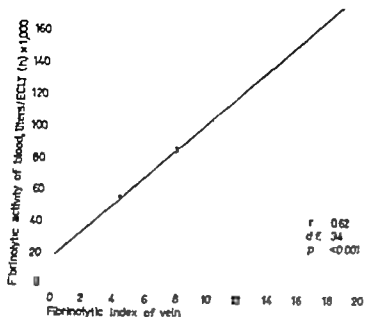


Fig. 2. Correlation of blood and vein wall fibrinolytic activities. Each dot represents a pair of simultaneous observations of the fibrinolytic activity in the blood and in the vein wall. Using linear regression method a correlation is obtained and the best possible line drawn from calculation.

during surgery. The mechanism of this phenomenon is thought to be due to the escape of plasminogen activator into the circulation [1] from vascularized tissue, such as muscle, in the wound. In our animal model there is very little trauma to the vascularized tissue and this may explain the absence of a rise in FA during surgery. Also, animals which were anesthetized for 3 h and received no surgery did not show any significant change in tissue FA [12] indicating that trauma was responsible for the observed changes.

Our findings of a linear relationship between the FA of the blood and the vein wall has not been previously reported. *Isacson and Nilsson* [8] studied a group of patients with chronic thrombophlebitis and found no correlation between the local FA in the blood and vein during venous occlusion. The reason may be that this group of patients have abnormal fibrinolysis and respond to an unphysiological stimulus with wide variations. *Browne et al.* [3] studied a group of patients with vascular diseases. Their results were suggestive of a correlation

between the blood and tissue FA, but owing to their inability to obtain a large number of measurements, statistical significance could not be reached. Our study using an animal model solves these problems and we are able to demonstrate a correlation with some degree of confidence. Such a relationship is likely to be present in the human since there is much similarity in fibrinolysis between man and the pig. This observation strengthens the hypothesis that the venous endothelium is the source of blood FA and its rate of release determines the spontaneous FA in the blood.

### Acknowledgement

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and vitamin B<sub>12</sub>-deficient marrow cells with <sup>14</sup>C-formate or <sup>14</sup>C-adenine for 24 h and found that the mean grain count over basophilic megaloblasts was higher than that over basophilic normoblasts by a factor of 2.5 with <sup>14</sup>C-adenine and 1.4 with <sup>14</sup>C-formate. In other relatively early studies, in which <sup>14</sup>C-adenine or <sup>14</sup>C-formate were used to investigate the pathophysiology of megaloblastic haemopoiesis, the design of the experiments was such that no comparisons could be made between the incorporation of these precursors into bone marrow cells from patients with normoblastic and megaloblastic erythropoiesis [9-12]. As the use of <sup>3</sup>H-thymidine for the study of DNA synthesis in vitamin B<sub>12</sub>- or folate-deficient cells may be criticised on the grounds that this radioactive precursor bypasses the block in the methylation of deoxyuridyate which is supposed to be the primary biochemical abnormality in these deficiency states, it is of interest to investigate the incorporation into DNA of other radioactive nucleic acid precursors which do not bypass this block. The present paper gives data on the incorporation of <sup>14</sup>C-glycine, <sup>14</sup>C-formate, <sup>3</sup>H-deoxyuridine and <sup>14</sup>C-adenine into both the DNA and RNA of bone marrow cells from haematologically normal and vitamin B<sub>12</sub>- or folate-deficient patients. In addition it gives data on the incorporation of <sup>3</sup>H-thymidine into DNA in marrow cultures containing higher concentrations of thymidine than have been generally used.

### Materials and Methods

Marrow aspirates from 18 haematologically normal patients with various diseases, 10 vitamin B<sub>12</sub>-deficient patients and 3 folate-deficient patients were studied. The haematologically normal patients showed normoblastic erythropoiesis and

had normal serum B<sub>12</sub> and red cell folate levels. Their marrow cells gave normal deoxyuridine suppressed values. The diagnosis of vitamin B<sub>12</sub> or folate deficiency was based on (1) the demonstration of megaloblastic haemopoiesis, (2) the results of serum vitamin B<sub>12</sub> and red cell folate levels measured by microbiological assay techniques using *Lactobacillus casei* and *Lactobacillus leichmannii*, respectively (3) the results of Schilling tests and (4) the results of deoxyuridine suppression tests performed on the marrow cells. All of the vitamin B<sub>12</sub>-deficient patients (with serum vitamin B<sub>12</sub> levels of 15-140 ng/l) had pernicious anaemia, and the folate-deficient patients (with red cell folate levels of 32-71 µg/l) suffered either from dietary deficiency (2 cases) or from coeliac disease (1 case).

Freshly-aspirated marrow was mixed with heparinised Hank's solution, and most of this mixture was used to prepare an erythrocyte-poor cell suspension containing about  $5.0 \times 10^6$  nucleated marrow cells per litre as described previously [10]. In some experiments this marrow cell suspension was used to determine the ideal concentration of thymidine for studies of the incorporation of this nucleoside into DNA. For this purpose several short-term cultures were set up each containing 1 ml of the cell suspension, 0.5 ml of autologous serum, 0.3 ml of Hank's solution, 0.1 ml of Hank's solution containing 0.5-10 µCi [6-<sup>3</sup>H]-thymidine (specific activity 25-26 Ci/mmol) and 0.1 ml of Hank's solution containing 0-60 µg non-radioactive thymidine. In the remaining experiments, cultures were set up by adding 1-ml aliquots of the marrow cell suspension to 0.5 ml of autologous serum, 0.4 ml of Hank's solution and 0.1 ml of Hank's solution containing one or other of the following: 10 µCi [6-<sup>3</sup>H]-thymidine (specific activity 25-26 Ci/mmol) together with 6 µg non-radioactive thymidine, 2 µCi [1-<sup>14</sup>C]-glycine (specific activity 59.5-60.7 mCi/mmol), 1 µCi deoxy-[6-<sup>3</sup>H]-uridine (specific activity 15 Ci/mmol) or 8 µCi [8-<sup>14</sup>C]-adenine (specific activity 58-61 mCi/mmol). The marrow cultures were incubated in shaking water bath at 37 °C for 1 h after which the radioactive cells were washed twice in ice-cold saline and resuspended in 1.2 ml of saline. Fractions containing macromolecular RNA and DNA were prepared by extracting the washed cells with perchloric acid (PCA) at different concentrations using method based on



## Incorporation of Radioactive Precursors into the Nucleic Acids of Bone Marrow Cells from Patients with Vitamin B<sub>12</sub> or Folate Deficiency<sup>1</sup>

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**Key Words.** Bone marrow DNA synthesis Folate deficiency Megaloblastic haemopoiesis RNA synthesis Vitamin B<sub>12</sub> Deficiency

**Abstract.** The *in vitro* incorporation of various radioactive precursors into the DNA and RNA of nucleated bone marrow cells was studied using marrow aspirates from patients with (1) normal blood counts and normoblastic marrows and (2) megaloblastic haemopoiesis due to vitamin B<sub>12</sub> or folate deficiency. Although earlier studies employing 0.01  $\mu$ M <sup>3</sup>H thymidine had indicated that the incorporation of <sup>3</sup>H thymidine was subnormal in some patients with vitamin B<sub>12</sub> or folate deficiency this finding was not seen with higher concentrations of thymidine. In fact, the rates of incorporation of <sup>3</sup>H-thymidine (0.22 and 12.5  $\mu$ M) as well as of <sup>14</sup>C-glycine, <sup>14</sup>C formate and <sup>14</sup>C-adenine into DNA were found to be abnormally high in some patients with megaloblastic haemopoiesis. By contrast, the incorporation of <sup>3</sup>H-deoxyuridine into DNA per 10<sup>4</sup> DNA-synthesising cells was found to be similar in the normoblastic and megaloblastic groups. Nevertheless, the ratios of the geometric mean for the incorporation of <sup>3</sup>H-deoxyuridine to the geometric means for the incorporation of the other radioactive precursors (all expressed as cpm per 10<sup>4</sup> DNA synthesising cells) were 2.34-3.92 times higher in the normoblastic than in the megaloblastic marrows giving direct support to the idea that there is some impairment of the methylation of deoxyuridylate in vitamin B<sub>12</sub> or folate-deficient marrows. No abnormality was detected in the rates of incorporation of <sup>14</sup>C-glycine, <sup>14</sup>C formate, <sup>14</sup>C-adenine and <sup>3</sup>H-deoxyuridine into RNA when the data were expressed as cpm per RNA absorbance unit.

There have been no recent studies comparing the incorporation of radioactive precursors other than <sup>3</sup>H-thymidine into the nucleic acids of bone marrow cells from

normal and vitamin B<sub>12</sub>- or folate-deficient individuals. In a paper published more than 20 years ago in which the technique of autoradiography was used to quantitate the incorporation of radioactive precursors, *Lajtha and Kumatori* [5] incubated normal

<sup>1</sup> This work was supported by the Medical Research Council.

Table I. Incorporation of various radioactive precursors into DNA of bone marrow cells from haematologically normal and megaloblastic patients

Radioactive precursor	Units of incorporation	Haematologically normal		Megaloblastic	
		geometric mean	95% confidence limits	geometric mean	95% confidence limits
<sup>3</sup> H-thymidine (0.22 $\mu$ M)	cpm/10 <sup>6</sup> cells	7,326	2,327-23,070 (7)	19,738**	2,879-135,333 (8)
	cpm/ $\mu$ g DNA	4,411	1,266-15,377 (7)	5,621	921-34,294 (8)
	cpm/10 <sup>6</sup> S-cells	51,750	20,284-132,026 (7)	88,721	16,145-487,567 (8)
<sup>3</sup> H-thymidine (12.5 $\mu$ M)	cpm/10 <sup>6</sup> cells	279	133-385 (12)	957*	125-7,332 (5)
	cpm/ $\mu$ g DNA	103	19-555 (12)	246	37-1,658 (5)
	cpm/10 <sup>6</sup> S-cells	1,630	634-4,191 (8)	4,642**	725-29,730 (5)
<sup>14</sup> C-glycine	cpm/10 <sup>6</sup> cells	134	61-284 (8)	422*	179-999 (5)
	cpm/ $\mu$ g DNA	45	10-192 (9)	111	43-280 (5)
	cpm/10 <sup>6</sup> S-cells	667	289-1,537 (8)	1,648*	320-3,226 (5)
<sup>14</sup> C-formate	cpm/10 <sup>6</sup> cells	97	55-172 (10)	225	73-701 (8)
	cpm/ $\mu$ g DNA	33	10-109 (12)	65*	15-280 (8)
	cpm/10 <sup>6</sup> S-cells	481	254-913 (8)	955**	255-3,578 (8)
<sup>14</sup> C-adenine	cpm/10 <sup>6</sup> cells	89	39-205 (11)	160**	40-648 (8)
	cpm/ $\mu$ g DNA	38	14-101 (12)	53	4-201 (8)
	cpm/10 <sup>6</sup> S-cells	424	171-1,501 (9)	721	135-721 (8)
H-deoxyuridine	cpm/10 <sup>6</sup> cells	1,216	383-3,868 (7)	1,151	490-2,704 (5)
	cpm/ $\mu$ g DNA	845	166-4,305 (7)	572	187-1,750 (5)
	cpm/10 <sup>6</sup> S-cells	6,261	2,038-19,239 (6)	4,543	1,740-11,861 (5)

p < 0.01    ■ < 0.05.

The number of patients studied is given within parentheses.

cpm per 10<sup>6</sup> S-cells. However whereas the differences between the means in the two groups were significantly different (p < 0.05 or < 0.01) for all the precursors (except <sup>3</sup>H-deoxyuridine) when the data were expressed as cpm per 10<sup>6</sup> nucleated cells, they were significantly different (p < 0.05 or < 0.01) only for <sup>14</sup>C-glycine and <sup>14</sup>C-formate when the data were expressed as cpm per  $\mu$ g DNA, and for <sup>3</sup>H-TdR (12.5  $\mu$ M) <sup>14</sup>C-glycine and <sup>14</sup>C-formate when the data

were expressed as cpm per 10<sup>6</sup> S-cells. Nevertheless, even in the case of <sup>3</sup>H-TdR (0.22  $\mu$ M) and <sup>14</sup>C-adenine, the incorporation (expressed as cpm per  $\mu$ g DNA or cpm per 10<sup>6</sup> S-cells) shown by some of the megaloblastic marrow samples was above the 95% confidence limits for the incorporation by the normoblastic group (fig. 2). The spread of values observed for the incorporation of <sup>3</sup>H-deoxyuridine into DNA in cpm per 10<sup>6</sup> S-cells and their 95% confidence

that described by *Felndegen et al.* [1]. For this purpose, 1 ml of the washed radioactive cell suspension was fixed with 1 ml of Carnoy's fixative (60% absolute ethanol, 30% chloroform and 10% glacial acetic acid) at room temperature for 10 min and then centrifuged at 1100 g for 5 min. The upper of the two layers formed after centrifugation was discarded, the volume of the lower layer was made up to 2 ml with 70% ethanol, and the mixture left overnight at 4 °C. The resulting precipitate was spun down at 1100 g for 5 min and the supernatant discarded. The precipitate was extracted with 2 ml of 2% PCA for 40 min at 4 °C to remove residual acid-soluble nucleotides and spun down again. The RNA was then extracted from the precipitate with 2 ml of 10% PCA for 5 h at room temperature. After separation of the RNA extract, the remainder of the precipitate was washed once with 2 ml of 10% PCA, and the supernatant after centrifugation was added to the RNA-containing fraction. Finally the DNA was extracted from the residual precipitate with 2 ml of 10% PCA for 2 h at 60 °C. The DNA and RNA extracts were stored at 4 °C until optical densities and radioactivity were measured.

The radioactivity in the extracts was measured after adding 0.5 ml of each extract to 9.5 ml of a water-miscible scintillation fluid (containing toluene and Triton X 100 in the proportion of 2:1, POPOP 0.2 g/l and PPO 4 g/l), using an LKB 1215 Rackbeta liquid scintillation counter. The DNA or RNA contents of the extracts were assessed by measuring their optical densities at 260 nm in a Unicam SP 500 spectrophotometer. In the case of the DNA extracts, the measured optical densities were converted to  $\mu\text{g}$  of DNA using the optical density given by the supernatant obtained after heating 10 mg calf thymus DNA (Sigma Chemical Co.) in 2 ml of 10% PCA to 60 °C for 2 h. The incorporation of radioactive nucleic acid precursors into DNA and RNA were expressed in three different ways: (1) cpm per  $\mu\text{g}$  DNA or cpm per RNA absorbance unit, (2) cpm in DNA or RNA per  $10^4$  nucleated cells and (3) cpm in DNA per  $10^4$  DNA-synthesizing cells (S-cells). The last expression was obtained by multiplying the uptake in cpm per  $10^4$  nucleated cells by 100/LI the LI (labelling index), which is the percentage of nucleated marrow cells engaged in DNA synthesis, was determined as described previously [10].

## Results

Figure 1 shows the relationship between the total concentration of thymidine (radioactive and non radioactive) in the marrow culture and the incorporation of the thymidine into DNA in two of the marrow samples studied. It is evident that concentrations less than  $1 \mu\text{M}$  were markedly rate-limiting for the incorporation of thymidine and that the incorporation curves were similar in shape in the normoblastic and vitamin  $B_{12}$ -deficient marrow samples.

The data on the incorporation of various radioactive compounds into the DNA of the nucleated bone marrow cells of haematologically normal and vitamin  $B_{12}$  or folate deficient patients are summarized in table I. The geometric means for the incorporation of all of the precursors except  $^3\text{H}$ -deoxyuridine were higher in the megaloblastic than in the normoblastic marrows irrespective of whether the data were expressed as cpm per  $10^4$  nucleated cells, cpm per  $\mu\text{g}$  DNA or

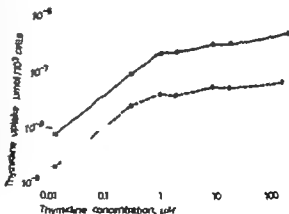


Fig. 1. Relationship between the concentration of thymidine in the marrow culture and the incorporation of thymidine into the DNA of the bone marrow cells from a haematologically normal patient (---) and a vitamin  $B_{12}$ -deficient patient with an Hb of 6.6 g/dl and an MCV of 114 fl (—).

Table II. Incorporation of various radioactive precursors into RNA of bone marrow cells from haematologically normal and megaloblastic patients

Radioactive precursor	Units of incorporation	Haematologically normal		Megaloblastic	
		geometric mean	95% confidence limits	geometric mean	95% confidence limits
<sup>14</sup> C-glycine	cpm/10 <sup>6</sup> cells	90	37-218 (9)	237*	96-584 (5)
	cpm/RNA				
	absorbance unit	378	141-1,016 (9)	524	205-1,341 (5)
<sup>14</sup> C-formate	cpm/10 <sup>6</sup> cells	51	22-119 (11)	110*	32-378 (8)
	cpm/RNA				
	absorbance unit	168	64-442 (11)	301	55-1,633 (8)
<sup>14</sup> C-adenine	cpm/10 <sup>6</sup> cells	1,135	515-2,300 (10)	1,600	610-4,202 (8)
	cpm/RNA				
	absorbance unit	4,491	1,953-10,317 (10)	3,599	936-13,836 (8)
<sup>3</sup> H-deoxyuridine	cpm/10 <sup>6</sup> cells	206	20-2,090 (7)	278	78-995 (5)
	cpm/RNA				
	absorbance unit	785	85-7,235 (7)	890	214-3,785 (5)

$p < 0.01$

The number of patients studied is given within parentheses.

Table III. Ratios of the incorporation of radioactive precursor into DNA to its incorporation into RNA in bone marrow cells from haematologically normal and megaloblastic patients

Patients	cpm per 10 <sup>6</sup> cells in DNA/cpm per 10 <sup>6</sup> cells in RNA (mean)			
	<sup>14</sup> C-glycine	<sup>14</sup> C-formate	<sup>14</sup> C-adenine	<sup>3</sup> H-deoxyuridine
Haematologically normal	1.560	2.216	0.101	8.126
Megaloblastic	2.219	2.775	0.112	4.386

No significant difference ( $p > 0.05$ ) was found for each precursor when the two patient groups were compared using Wilcoxon's rank sum test.

the *in vitro* incorporation of <sup>3</sup>H-TdR into the DNA of bone marrow cells from patients with vitamin B<sub>12</sub> or folate-deficient megaloblastic haemopoiesis. Both in normal and megaloblastic marrows the incorporation of thymidine into DNA was found to be strongly dependent on the con-

centration of thymidine in the culture when the concentration was below 1  $\mu$ M. This phenomenon is probably at least partly dependent on the fact that bone marrow cells and particularly the neutrophil granulocytes are known to degrade thymidine *in vitro* [2, 6, 7].

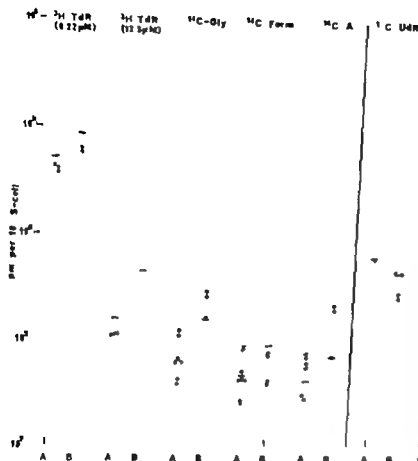


Fig. 2. Incorporation of various radioactive compounds into DNA expressed as cpm per  $10^6$  S-cells. A = Haematologically normal patients; B = vitamin B<sub>12</sub> or folate-deficient patients; TdR = thymidine Gly = glycine Form = formate A = adenine, and UdR = deoxyuridine. The geometric means and 95% confidence limits are indicated by the horizontal bars.

limits were similar in the normoblastic and megaloblastic groups (fig 2)

There appeared to be no correlation between the haemoglobin levels or MCVs of the patients with megaloblastic haemopoiesis and the rates of incorporation into DNA (expressed as cpm per  $10^6$  S-cells) of  $^3\text{H}$ -TdR,  $^{14}\text{C}$ -glycine  $^{14}\text{C}$ -formate or  $^{14}\text{C}$ -adenine.

Table II gives the data on the incorporation of various radioactive compounds into the RNA of the nucleated bone marrow cells in the two groups of patients studied. It can be seen from the table that the geometric means for the incorporation of  $^{14}\text{C}$ -glycine and  $^{14}\text{C}$ -formate but not of  $^{14}\text{C}$ -adenine or  $^3\text{H}$ -deoxyuridine were significantly higher ( $p < 0.01$ ) in the megaloblastic than in

the normoblastic marrows when the data were expressed as cpm per  $10^6$  cells. By contrast, no statistically significant differences ( $p > 0.05$ ) were found between the megaloblastic and normoblastic groups with any of the four precursors studied when the data were expressed as cpm per RNA absorbance unit. In addition, the DNA:RNA incorporation ratios for various radioactive compounds were not significantly different ( $p > 0.05$ ) in these two groups (table III).

### Discussion

The data reported here show that rate-limiting concentrations of thymidine have been used in most of the earlier studies of

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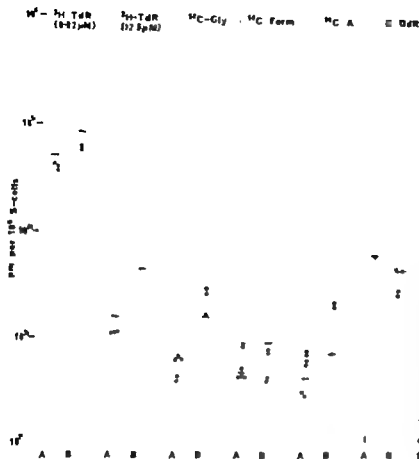


Fig. 2. Incorporation of various radioactive compounds into DNA expressed as cpm per  $10^6$  S-cells. A = Haematologically normal patients; B = vitamin B<sub>12</sub> or folate-deficient patients; TdR = thymidine; Gly = glycine; Form = formate; A = adenine, and UdR = deoxyuridine. The geometric means and 95% confidence limits are indicated by the horizontal bars.

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Table II gives the data on the incorporation of various radioactive compounds into the RNA of the nucleated bone marrow cells in the two groups of patients studied. It can be seen from the table that the geometric means for the incorporation of  $^{14}\text{C}$ -glycine and  $^{14}\text{C}$ -formate but not of  $^{14}\text{C}$ -adenine or  $^3\text{H}$ -deoxyuridine were significantly higher ( $p < 0.01$ ) in the megaloblastic than in

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### Discussion

The data reported here show that rate-limiting concentrations of thymidine have been used in most of the earlier studies of

ing that megaloblastic marrows suffer from some impairment of the incorporation of  $^3\text{H}$ -deoxyuridine into DNA (and hence of the methylation of deoxyuridylylate) relative to the incorporation of the other DNA precursors. From the geometric means expressed in cpm per  $10^6$  S-cells given in table I, it can be calculated that the ratios of the incorporation of  $^3\text{H}$ -deoxyuridine to the incorporation of  $^3\text{H}$ -TdR (0.22  $\mu\text{M}$ ),  $^3\text{H}$ -TdR (12.5  $\mu\text{M}$ ),  $^{14}\text{C}$ -glycine,  $^{14}\text{C}$ -formate and  $^{14}\text{C}$ -adenine were, respectively 2.37, 3.92, 3.40, 2.74 and 2.34 times higher in the normoblastic than in the megaloblastic group.

Statistically significant differences were not found between the megaloblastic and normoblastic groups either in the rate of incorporation of radioactive precursors into RNA, expressed as cpm per RNA absorbance unit, or in the DNA:RNA incorporation ratios.

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Table 1. Haematological data and plasma erythropoietin level in 12 anemic patients

No.	Diagnosis	Haemo- globin g/dl	Haemato- crit %	Reticulo- cytes %	Coombs test	Iron $\mu$ g/dl	Hapto- globin mg/dl	Ep U/ml plasma
1	Iron deficiency anaemia	5.9	21	1.3	0	23	130	6.7
2	Iron deficiency anaemia	10.8	37	0.5	0	104	100	5.6
3	Iron deficiency anaemia	8.8	32	0.3	0	4	74	5.17
4	Iron deficiency anaemia	11.5	36	1.3	0	34	0	3.6
5	Iron deficiency and megaloblastic anaemia	8.5	28	6.5	II	66	—	5.4
6	Iron deficiency anaemia	10.2	32	0.3	0	39	160	7.0
7	Iron deficiency anaemia	10.4	33	0.6	0	8	215	12.5
8	Iron deficiency anaemia	10.0	32	0.5	0	47		2.2
9	Autoimmune haemolytic anaemia	8.9	25	2.9	direct 0 indirect +	107	0	10.8
10	Haemolytic anaemia	10.5	32	1.9	direct + indirect 0	80	0	14.64
11	Haemolytic anaemia	9.5	30	2.4	direct $\pm$ indirect 0	99	90	4.4
12	Iron deficiency anaemia	10.1	29	1.1	0	23	100	5.2
Mean $\pm$ SD								6.93 $\pm$ 1.53

The plasma obtained after centrifugation was divided into tubes containing 0.4 ml and stored at 20 °C for no longer than 1 month. Each plasma was examined twice in duplicates.

**Animals and Cells.** C<sub>57</sub>BL/6J mice, hormonally primed and mated according to the method of Leachard *et al.* [29], were used. 13-day embryonic livers were removed, placed in Eagle's minimal essential medium (MEM, Gibco, Grand Island, N.Y.), containing 1% penicillin and streptomycin, and kept at 4 °C in air with 5% CO<sub>2</sub>. Pooled livers were cut in small pieces and cell suspension was obtained by repeatedly squeezing the tissue

through siliconeized, flacetipped Pasteur pipette, as previously described [3], except that the liver fragments were not trypsinized.

**RNA Synthesis.** 1.0 ml of cell suspension containing  $2 \times 10^6$  cells/ml of culture medium was incubated for 2 h with and without the patients' and controls' plasma at 37 °C without agitation in humidified atmosphere containing 5% CO<sub>2</sub>. 3  $\mu$ Cl of 5-<sup>3</sup>H-uridine (spec. act. 5 Ci/mmol, Nuclear Research Center Negev Beer-Sheva, Israel) were added for additional 60 min. The reaction was stopped by addition of 9.0 ml cold 0.9% sodium chloride solution, the cells are thoroughly mixed,

## Quantitative Determination of Human Plasma Erythropoietin Using Embryonic Mouse Liver Erythroblasts

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**Key Words.** Erythroblasts Human plasma erythropoietin Mouse liver

**Abstract.** An *in vitro* bioassay for the quantitative detection of erythropoietin (Ep) in human plasma is described. The method is based on the increased  $^3\text{H}$ -uridine incorporation into 12-day embryonic mouse liver erythroblasts due to Ep. It was found that 11 out of 12 anaemic patients showed high plasma Ep level, while 7 out of 8 patients suffering from polycythaemia revealed low Ep values. In 4 out of 8 patients with chronic renal failure the Ep level was within the normal range, whereas in the remaining 4 it was higher than normal. The relative simplicity of the method and the reproducibility of the results make it useful even in the routine laboratory.

### Introduction

Erythropoietin (Ep) stimulates RNA synthesis in cultured fetal mouse liver erythroid cells [25]. According to Djaldetti *et al.* [6] the stimulation of RNA synthesis is a result of a selective effect of Ep on the proerythroblasts derived from 12-day embryonic mouse livers, although it was also apparent on 11 and 13-day embryonic liver erythroblasts. Recently Dunn *et al.* [7, 8] demonstrated that erythroid stimulating factor (ESF) activity of human serum can be detected by the determination of  $^{59}\text{Fe}$  incorporation into haem in 13- to 14-day fetal mouse liver cells.

The present work provides a bioassay for the determination of Ep concentration in

human plasma, by measuring the enhancement of  $^3\text{H}$  uridine incorporation into RNA of 12-day fetal mouse liver erythroid cells, caused by the plasma.

### Materials and Methods

#### *In vitro* Ep Bioassay

**Patients.** Plasma erythropoietic activity was measured in 12 anaemic patients (table I), 8 patients suffering from polycythaemia (table II), and 8 patients suffering from chronic renal failure. Their serum urea level ranged between 130 and  $\sim 60$  mg/dl, and the haemoglobin value between 6.5 and 12.5 g/dl. 21 healthy volunteers served as controls.

**Plasma.** 5 ml of venous blood were withdrawn with a heparinized syringe, from patients and controls (haemoglobin level between 13 and 15 g/dl).

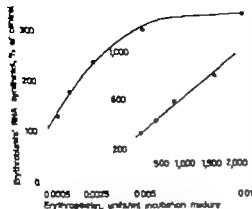


Fig. 1. Dose-response relationship between Ep concentration and erythroblasts' RNA synthesis. The lower represents the same relationship expressed as double reciprocal curve.

eroblastic anaemia). The haemoglobin of the anaemic patients ranged between 7.6 and 9.3 g/dl. In addition, 7 patients with polycythaemia were (haemoglobin 17.5–19.6 g/dl) were examined. Plasma was withdrawn as described above and stored at 20 °C.

## Results

### In vitro Ep Bioassay

**Ep Effect on RNA Synthesis.** A dose response relationship between Ep concentration and  $^3\text{H}$ -uridine incorporation in the erythroid cells is shown in figure 1. The minimal detectable dose of Ep was 0.0005 IU/ml of culture.

**Plasma Ep Level.** The mean Ep level in the plasma of 21 healthy subjects was  $2.12 \pm 0.84$  U/ml (mean  $\pm$  SD range 1–4 U/ml).

**Anaemic Patients** 10 out of 12 anaemic patients examined (table I) showed plasma Ep levels significantly higher than the controls ( $p < 0.001$ ). The mean Ep concentra-

tion in their plasma was found to be  $6.93 \pm 1.03$  U/ml, ranging between 4.4 and 14.64 U/ml. 1 patient suffering from iron deficiency anaemia had the Ep level within the normal range (2.2 U/ml).

**Polycythaemic Patients.** The mean value of the Ep level in 7 out of 8 polycythaemic patients was  $1.069 \pm 0.25$  U/ml with a range of 0.74–1.42 U/ml, i.e., significantly lower in comparison to normal subjects ( $p < 0.01$  table II). In 5 patients the Ep value was at the lower limit of normal.

**Chronic Renal Failure Patients.** The plasma Ep in 4 out of 8 patients with chronic renal failure was within the normal values mean  $2.27 \pm 1.27$  U/ml, with a range of 1–3.23 U/ml. In the other 4 patients, a significant increase of the Ep level was observed ( $p < 0.001$ ), mean  $7.86 \pm 1.08$  U/ml, with a range of 6.45–9.08 U/ml plasma. Uræmic patients examined before and after dialysis did not show changes in the plasma Ep level.

### In vivo Ep Bioassay

**Plasma Ep Level.** The Ep level in the plasma of 13 healthy subjects was  $25.8 \pm 11$  mU/ml (mean  $\pm$  SD range 14–38 mU/ml). The Ep concentration in the plasma of the anaemic patients was  $80 \pm 52$  mU/ml (mean  $\pm$  SD range 32–180 mU/ml), a value significantly higher than in the healthy subjects ( $p < 0.001$ ). In the 7 patients with polycythaemia, the mean Ep level was 18–8 mU/ml (range 11–30 mU/ml), a lower but not statistically significant, value as compared with the normal one ( $p < 0.1$ ).

## Discussion

The present study provides a relatively simple *in vitro* assay for the quantitative de-



Table II. Haematological data and plasma Ep level in 8 patients with polycythaemia

No.	Haemo- globin g/dl	Haemato- crit %	Reticu- lyocytes %	Iron µg/dl	Vit B <sub>12</sub> pg/ml	LAP score	WBC/ mm <sup>3</sup>	Platelet/ mm <sup>3</sup>	ESR mm/h	RBC vol., ml/kg	Plasma vol., ml/kg	Ep U/ml plasma
1	15.3	47	0.7	80	519	103	9,300	325,000	8/18	33.8	39.7	1.0
2	17.4	52	1.0	-	712	60	7,200	317,000	-	-	-	1.0
3	16.5	48	1.0	-	783	140	4,200	200,000	1/5	31.2	33.8	1.42
4	17.0	50	0.8	70	409	154	7,600	345,000	3/7	33.3	37.6	1.29
5	16.8	53	0.3	100	473	33	6,300	324,000	2/6	35.3	39.9	1.12
6	17.6	57	0.4	91	-	■	5,500	268,000	1/4	31.0	35.0	0.88
7	16.6	49	0.9	107	-	■	8,400	315,000	4/13	34.4	45.7	0.74
8	15.8	50	1.3	40	476	-	9,300	460,000	-	53.0	40.0	2.3 <sup>1</sup>
Mean ± SD												1.069 ± 0.25

LAP = Leukocytic alkaline phosphatase.

<sup>1</sup> Not included in the mean value.

centrifuged and the macromolecules precipitated in 2.0 ml of 10% trichloroacetic acid. The precipitate was kept overnight at 4 °C and filtered on fibreglass filters (GF/C, Tamar Jerusalem, Israel). The filters were dried for 15 min at 60 °C, placed in glass vials (Packard Instruments, Downers Grove, Ill.) containing a toluene scintillation mixture consisting of 0.01% 1,4-bis-(2-(4-methyl-5-phenyl-oxazolyl)) benzene and 0.4% 2,4-diphenyl-oxazol (Packard) and the radioactivity counted in a Tricarb liquid scintillation spectrometer (Packard, Model 3390). Samples in which variations between duplicates exceeded 10% were disregarded.

**Standard Ep Curve** Ep (step-3 CMRL, freeze-dried, prepared from phenylhydrazine-treated sheep, Connaught Laboratories, Willowdale, Ont., Canada) was dissolved in culture medium at different concentrations. The incubation procedure was the same as described above. <sup>3</sup>H-methionine incorporation into RNA of erythroid cells incubated with Ep was calculated as percentage of radioactivity (cpm) compared with unstimulated cells. The dependence of RNA synthesis upon Ep concentration was plotted according to the Lineweaver-Burk 'double reciprocal' curve (fig. 1).

**Plasma Ep.** Patients' and controls plasma Ep values were determined in four different plasma concentrations, prepared by twofold dilutions of the plasma in culture medium, the highest of which did not exceed 0.5% (5 µl/ml culture). The

radioactivity found for each plasma dilution was calculated as percentage compared with cultured erythroblasts without plasma. Ep values were derived from the standard Ep curve (fig. 1) prepared for each experiment. The final result was expressed as the mean of two consecutive plasma concentrations.

#### In vivo Ep Bioassay

Erythropoietic activity in the plasma was determined by using the posthypoxic polycythaemic mouse assay [9]. 6-week-old virgin female B6F mice were injected intraperitoneally with 5 mg of Imferon and kept for 3 weeks in a closed chamber in an atmosphere of 6% oxygen and 92% nitrogen. 7 and 8 days after removal from the hypoxic environment, 1.0 ml of saline, 3 standard levels of 0.4, 0.2 and 0.1 units of Ep or test plasma were injected intraperitoneally. On the next day <sup>59</sup>Fe citrate (0.5 µCi/0.2 ml, Amersham, England) was injected intravenously and 48 h later the animals were bled by heart puncture. Erythropoietic activity was related to the uptake of <sup>59</sup>Fe into circulating erythrocytes. Each plasma was tested in a group of 3-6 mice. Samples from animals with haematocrit level lower than 50% were eliminated.

Plasma erythropoietic activity was measured in 13 healthy human subjects (haemoglobin 13-15 g/dl), as well as in 7 patients with anaemia (5 with iron deficiency 1 with haemolytic, and 1 with id-

*in vitro* Ep stimulation are: shorter periods of incubation, avoidance of fetal calf serum, and use of lower amounts of human plasma. The results on the plasma Ep level obtained in this study correlated well with the patients' diagnosis.

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lection of Ep activity The activity of this hormone in human plasma is measured indirectly as a function of  $^3\text{H}$ -uridine incorporation into the RNA of erythroid cell precursors derived from fetal mouse livers at the 12th day of gestation.

Fetal mouse liver by days 11 and 14 of gestation provides a cell population consisting of erythroid cells [21] Previous experiments have demonstrated that Ep stimulates the RNA synthesis in cultured 12-day fetal mouse liver proerythroblasts [6] as measured by radioactive uridine incorporation, while the effect of the hormone on more mature red cell precursors and on hepatic cells was not significant. Using a curve, relating various concentrations of standard Ep to the  $^3\text{H}$ -uridine uptake it is possible to estimate the Ep concentration per milliliter of examined plasma.

The Ep level in control human plasma in the present study was found to be in the range of 1-4 IU/ml. These Ep concentrations are higher than the values reported by other investigators [8 13 17 18 20 22] On the other hand, extrapolation from the data given by Von Preyss and Goudsmit [30] revealed similar values in normal human serum The discrepancy between the Ep levels obtained by using different methods raises the question about the identity of the substance being assayed by each method. The Ep levels determined by the *in vivo* bioassay corresponded well to the values reported by Jordan *et al.* [17] Kazal and Erslev [18] and De Klerk *et al.* [3] but were much lower than those obtained in the *in vitro* bioassay

The assay tested on patients with different diseases gave satisfactory results. Plasma Ep level determined in patients suffering from different types of anaemia was significantly higher as compared to normal con-

trols High Ep level was reported in patients with iron deficiency [15] aplastic [15, 19 26] megaloblastic [26] refractory [23] and haemolytic anaemia [14 16]

The plasma of 4 out of 8 patients with chronic renal failure and low haemoglobin level showed normal Ep value, while the other 4 showed a significant increase. Some of the patients suffering from chronic renal failure and uraemia are known to have a decreased level of plasma Ep, despite the anaemia [10 11] others have normal or high Ep levels [1 12, 24 28] The majority of the polycythaemic patients possessed a low level of Ep activity as was expected.

Recently Dunn *et al.* [8] introduced a method for the measurement of Ep in untreated human sera by using the incorporation of radioactive iron into fetal mouse liver cells. Later on, it was shown that a wide variety of serum constituents may influence the Ep-mediated stimulation of haem synthesis [2, 27] Serum iron [2] iron transferin concentration in the serum samples and serum concentration in the culture [27] were found to influence  $^{59}\text{Fe}$  incorporation into haem. Therefore, a modification of the mouse fetal liver cell bioassay was suggested, consisting in the correction of these factors effect and the use of internal standard. On the other hand, De Klerk *et al.* [4] have shown a toxic effect of human serum on mouse erythroblasts due to complement-dependent heteroantibodies. A similar toxicity expressed as lower stimulation of the erythroblasts RNA synthesis was found in the present study when higher concentrations of plasma were used. It is conceivable therefore that the low Ep level found by other investigators was most probably due to a toxic effect or to inhibitor(s) present in the plasma and sera.

The advantage of the present method for

*in vitro* Ep stimulation are: shorter periods of incubation, avoidance of fetal calf serum, and use of lower amounts of human plasma. The results on the plasma Ep level obtained in this study correlated well with the patients diagnosis.

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## Agglutination of Granulocytes from Chronic Myeloid Leukaemia by Concanavalin A

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**Key Words.** Agglutination · Chronic myeloid leukaemia · Concanavalin A

**Abstract.** Peripheral blood leucocytes from chronic myeloid leukaemia (CML) patients previously untreated or in relapse are agglutinated by concanavalin A (Con A) at concentrations of 25-400 µg/ml. Leucocytes from normal subjects, from CML patients in remission and from patients with granulocytosis are not agglutinated by Con A. Normal bone marrow cells which contain immature and mature myeloid cells are not agglutinated either by Con A. These results suggest that an alteration occurs in the cell surface of myeloid cells in CML due to leukaemic change and results in the agglutination of these cells by Con A. If peripheral blood leucocytes of CML patients in relapse are separated into immature and mature fractions, both these fractions are agglutinated by Con A suggesting that this alteration occurs in the cell surface of both the immature and the mature myeloid cells.

### Introduction

Some of the well-documented differences between normal and malignant cells reside in their cell membranes [15]. While normal cells are not agglutinated by lectins such as concanavalin A (Con A) and wheat germ agglutinin, malignant cells obtained from transplanted tumours or cells transformed *in vitro* are agglutinated [8, 9, 15].

Among the human tumours, malignant cells of lymphoid origin such as those from patients with chronic lymphatic leukaemia, lymphomas, Hodgkin's disease, and Burkitt's lymphoma have been shown to differ from normal human lymphocytes on the ba-

sis of their agglutination by Con A and by their ability to redistribute labelled Con A on their cell surface differentially [2-5, 10, 11, 13, 14]. In the present paper we report differences between normal granulocytes and the cells of granulocytic series from patients with chronic myeloid leukaemia (CML) on the basis of their agglutination by Con A.

### Material and Methods

Highly purified soluble Con A obtained from Biochemicals unit, V.P. Chest Institute, New Delhi [16], was further purified by the method of Bhargava et al. [6]. The agglutinating capacity of Con A was checked with Yoshida sarcoma cells which were agglutinated at 5 µg/ml Con A. *n*-Methyl



mannoside was obtained from Sigma Chemicals, St. Louis, Mo. All the glassware used was siliconized with Siliclad (Clay Adams, Parsippany NJ.).

Peripheral blood was collected in tubes containing 5 IU/ml preservative free heparin. Leucocytes from CML patients with high leucocyte counts were separated by sedimentation at room temperature for 45 min. The buffy coat was removed from the peripheral blood of following groups of patients: (1) CML patients in remission, (2) patients with granulocytosis, and (3) normal subjects. It was suspended for 3-4 min in 0.83% ammonium chloride buffered with Tris-HCl at pH 7.2 to lyse the erythrocytes [7]. The cell suspension was diluted with phosphate-buffered saline free of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  (PBS) and centrifuged at 800 rpm. All the leucocyte suspensions were washed thrice and finally suspended in PBS. Bone marrow aspirates were also processed similarly.

#### Separation of Mature and Immature Cells.

The mature and immature cells from peripheral blood of CML patients in relapse were separated by a method modified from that of McCulloch [12]. Peripheral blood leucocytes were incubated in 70-mm Falcon plastic dishes at a density of  $1 \times 10^6$  cells per dish in Dulbecco's medium supplemented with 15% human AB group serum. After 2 h, the supernatant from the dishes containing non-adherent cells was removed. The cells adherent to dishes were washed thrice with PBS and incubated for 10 min with 2 ml of saline EDTA and detected by light pipetting. This adherent population was found to consist of more than 90% mature granulocytes and some bands while the non-adherent population consisted of immature cells. Only samples with more than 90% viable cells, as tested by dye exclusion using 0.4% erythrocin B, were used.

#### Agglutination Assay

Con A was dissolved freshly for each experiment. 0.1 ml of cell suspension at a density of  $4 \times 10^6$  cells/ml was added to 0.1 ml of Con A solution at a final concentration of 0, 25, 50, 100, 200 and 400  $\mu\text{g/ml}$ . The mixture was incubated at 37 °C for 10 min and mixed in a vortex mixer for 15 sec to break loose aggregates. The suspension was examined microscopically in a haemocytometer and agglutination was scored according to Imber and Sachs [9] as follows: no clustered cells in suspension, which took into account an occasional

aggregate present in controls; + up to 30% cells agglutinated, with clusters of 3-4 cells; ++ 30-60% cells agglutinated, with aggregates of 10-15 cells; +++ 60-90% cells agglutinated, with aggregates of 15 or more cells.  $\alpha$ -Methyl mannoside (0.1 M) completely inhibited agglutination.

Clinical evaluation of patients and haematological studies were carried out by the haematologist, Tata Memorial Hospital.

## Results

Peripheral blood leucocytes from 9 normal subjects were not agglutinated by Con A up to 400  $\mu\text{g/ml}$  (table I). Moreover bone marrow cells from 11 normal subjects were not agglutinated by Con A (table I).

Peripheral blood leucocytes were collected from CML patients either at initial diagnosis or during relapse before starting the therapy. These patients had high total leucocyte counts and immature myeloid cells in their peripheral blood. Table II gives the case number, the Con A agglutination index and the total and differential leucocyte counts of CML patients. Leucocytes from all the 23 patients studied were agglutinated at 50  $\mu\text{g/ml}$  and higher concentrations of Con A. In 16 patients agglutination was observed at 25  $\mu\text{g/ml}$  Con A. Since there was

Table I. Agglutinability of human leucocytes from different sources by Con A

Source of cells	Number of samples	Agglutination by Con A (25-400 $\mu\text{g/ml}$ )
Normal peripheral blood leucocytes	9	nil
Normal bone marrow cells	11	nil
Leucocytes from patients with granulocytosis	7	nil

Table II. Agglutination of peripheral blood leucocytes of CML patients in relapse by Con A

Case No.	Aggregates		WBC count									
	Con A concn- trations, $\mu\text{g/l}$		total	$10^9/\text{mm}^3$	differential							
	25	50-400			Blt	Pro and Myel	Met Myel	Pol	Lymph	Eos	Mono	
AB-9160 <sup>1</sup>	+++	+++	248.0		3	20	26	41	8	2	0	
AD-5122 <sup>2</sup>	+++	+++	25.0		1	12	16	61	7	2	1	
AH-718 <sup>1</sup>	+	+	92.0		2	32	27	27	8	2	0	
AF 5884 <sup>1</sup>	-	+	320.0		0	30	7	49	2	2	0	
AD-14073	-	++	240.0		2	30	28	30	8	2	0	
AD-6870	-	++	14.7		94	0	0	0.5	5	0.5	0	
AF-6560	-	+	80.0		0	12	22	56	8	1	5	
AF-6589	-	++	24.0		2	26	30	30	6	2	0	
AF 10324	-	++	168.0		1	20	24	47	5	3	0	
AF-11864	++	+++	232.0		3	39	0	30	5	2	0	
AF 1451	+++	+++	170.0		1	33	27	29	8	0	2	
AF 13032	++	+++	198.0		1	29	26	38	6	0	0	
AF-13598	+++	+++	260.0		0	23	27	34	8	2	0	
AG-715	+++	+++	560.0		1	42	37	13	15	1	0	
AG-818	++	+++	130.0		0	24	28	42	6	0	0	
AF-9899	++	+++	58.8		0	2	4	69	20	2	0	
AF 10069	++	++	112.0		0	13	25	52	9	0	0	
AD-14143	+++	+++	320.0		2	42	26	23	7	0	0	
AF-12182	+++	+++	120.0		1	29	21	33	11	2	3	
AE 5236	+++	+++	86.0		0	36	25	34	5	0	0	
AE-881	+++	+++	158.0		8	24	21	37	8	2	0	
AH-541	+	+	320.0		2	37	30	28	4	5	0	
AC 13403	-	+++	486.0		2	18	22	49	7	2	0	

Blt = Blast Pro and Myel = promyelocytes and myelocytes Lymph = lymphocytes Met = metamyelocytes  
Pol = polymorphs Eos = eosinophils, Mono = monocytes.

Patients followed-up in remission: table III.

little difference in the degree of agglutination at 50  $\mu\text{g/ml}$  Con A and higher concentrations, the results have been grouped together in the tables.

Leucocytes from 11 CML patients in clinical and haematological remission were not agglutinated by Con A (table III). Patients were considered to be in remission when all clinical signs of disease disappeared and leucocyte count was less than

15,000  $\text{mm}^3$  with normal differential counts. Agglutination studies were carried out on remission patients' cells at least 7 days after chemotherapy had been stopped. Leucocytes from 5 of these patients had agglutinated when the patients were in relapse, but agglutination was not observed during remission (No. AB-9160, AD-5122, AF-5884 and AH 718, tables II and III AC 169 tables III and IV).

Table III. Agglutinability of peripheral blood leucocytes from CML patients in remission by Con A

Case No	Aggregates Con A concentrations, $\mu\text{g/ml}$		Differential count							
	25	50-400	total WBC $\times 10^3/\text{mm}^3$	Blt	Myel	Met	Pol	Lymph	Eos	Mono
AB-9160 <sup>1</sup>	-	-	5.9	0	0	0	71	25	2	2
AF-5384 <sup>1</sup>	-	-	6.2	0	0	0	70	25	2	3
AD-5122 <sup>1</sup>	-	-	12.4	0	0	0	70	29	0	1
AH 718 <sup>1</sup>	-	-	12.4	0	0	0	70	18	4	4
AC 169 <sup>2</sup>	-	-	11.4	0	0	0	69	25	3	3
AE-12873	-	-	5.2	0	0	0	76	19	2	2
AF-5136	-	-	10.6	0	0	0	75	14	2	2
AH 16147	-	-	7.6	0	0	0	77	15	3	5
AH-15102	-	-	8.7	0	0	0	74	20	3	3
AF-12451	-	-	7.4	0	0	0	81	30	4	5
AJ-9669	-	-		0	0	0	67	26	4	3

See legend, table II.

<sup>1</sup> Patients studied in relapse - table II.<sup>2</sup> Patient studied in relapse - table IV

Table IV. Agglutination of total leukaemic leucocytes and cells separated into adherent and non-adherent fractions from peripheral blood of CML patients in relapse, by Con A

Case No.	Con A <sub>5</sub> 50-400 $\mu\text{g/ml}$			Differential count							
	total cells	adherent cells	non-adherent cells	total WBC $\times 10^3/\text{mm}^3$	Blt	Pro and Myel	Met	Pol	Lymph	Eos	Mono
AH 12664	++	++	+++	150.0	1	19	26	40	9	5	0
AG-13109	+++	+++	+++	265.0	3	36	34	23	4	0	0
AH-13118	++	+	++	264.0	6	24	35	29	6	0	0
AH 13347	++	++	++	284.2	5	24	25	35	0	3	B2
AG-1537	++	++	++	240.0	1	29	32	31	5	2	0
AH 13966	+++	++	+++	216.0	1	39	25	27	5	3	0
AH 13966 (R)	++	++	+++	50.2	4	14	19	54	6	1	B2
AF-13711	++	++	+++	54.0	0	15	12	63	9	0	1
AJ-8175	++	++	++	200.0	1	20	54	25	0	0	0
AJ 7745	++	++	++	610.0	3	46	22	20	8	1	0
AJ-7195	++	++	++	160.0	0	20	23	43	6	0	2
AC-169	+	+	+	40.0	0	12	16	57	10	3	2
AJ-7269	+	+	+	290	16	30	10	30	5	6	-

See legend, table II, B = Basophils.

Leucocytes collected from 7 patients with granulocytosis were not agglutinated by Con A (table I). These patients had infections, abscesses and showed elevated total leucocyte and granulocyte counts in their peripheral blood ranging from 10,000 to 25,000/mm<sup>3</sup>.

In the above studies total leucocytes from CML patients in relapse were used for the assay. It was, therefore, essential to know whether only the mature leucocytes, the immature cells, or both these fractions, were agglutinated by Con A. The total leucocytes were separated into mature and immature cells and assayed separately for agglutination. The differential counts of these fractions are not given since the experiments were carried out mainly on highly enriched populations of mature granulocytes, and their purity for each sample was checked microscopically. The total unseparated leucocytes as well as both separated fractions were agglutinated by Con A in all cases (table IV).

## Discussion

Differences between the plasma membranes of normal and malignant cells of human lymphoid origin have been demonstrated with the use of Con A as a probe [2-5, 10, 13, 14]. These alterations include agglutination of the malignant cells by Con A and alteration in the pattern of redistribution of Con A probe on the plasma membrane, when compared to the normal cells. Our studies indicate that similar alterations occur in the cell surface of myeloid cells in CML. While the leucocytes from CML patients in relapse were agglutinated by Con A, those from normal subjects and CML patients in remission were not agglutinated.

This is clearly brought out from our results in 5 patients wherein the leucocytes were agglutinated by Con A when the patients were in relapse, but not when the patients entered haematological remission. Our studies on patients with granulocytosis show that when the peripheral blood granulocytes are increased due to non-malignant conditions, i.e. in response to infections, the leucocytes do not exhibit this alteration.

Con A has been shown to agglutinate embryonic and fetal cells [1]. The leucocytes from the peripheral blood of CML patients in relapse contain mature granulocytes as well as immature myeloid cells. Cells of comparable immature status are present in the normal bone marrow. The non-agglutinability of such immature cells from normal marrow rules out the possibility that the agglutination of leucocytes of CML patients in relapse is due to the occurrence of immature cells and suggests that this must be due to their leukaemic state. The mature granulocytes from CML patients in relapse are morphologically indistinguishable from normal granulocytes, but they agglutinate with Con A, whereas the normal mature granulocytes do not. These observations suggest that both the mature and immature leucocytes from peripheral blood of CML patients undergo an alteration in the plasma membrane. When the patients enter haematological remission, such leucocytes with altered surface are not detected in the peripheral blood with the use of Con A agglutination assay. Such cells with membrane alteration might have disappeared from the peripheral blood in CML patients during remission or are too few to be detected by agglutination assay. They reappear during relapse. Ben-Barat *et al.* [5] have also observed a similar pattern in chronic lymphatic leukaemia.

Our studies thus indicate, that, as in various lymphoid malignancies, in CML also, the cells of the granulocytic series are agglutinated by Con A. Leucocytes from CML patients in remission from normal subjects, from patients with granulocytosis and normal immature myeloid cells from the bone marrow are not agglutinated by Con A. This alteration in the cell surface of the myeloid cells in CML patients occurs in immature cells as well as mature granulocytes.

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## Response to Therapy as Prognostic Factor in Chronic Lymphocytic Leukemia

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**Key Words.** Chronic lymphocytic leukemia. Prognosis. Therapy

**Abstract.** In 56 patients with chronic lymphocytic leukemia the value of clinical staging and response to therapy were analyzed. 51 patients were given therapy. Actuarial survival curves for patients who showed a complete or partial remission differed significantly for patients who either improved or in whom therapy failed ( $p < 0.001$ ). The 5-year survival fraction was significantly better determined by response to therapy than by clinical staging. 5-year survival fraction for stage 0 + I + II was 59%, and for III + IV 27% (not significant,  $p = 0.08$ ). 5-year survival fraction for patients with failure or only improvement to therapy was 20%, and with complete or partial remission 72% (significant,  $p = 0.003$ ).

### Introduction

The prognosis in chronic lymphocytic leukemia (CLL) varies considerably. Some patients succumb within 1 year whereas others may survive for more than 10 years after diagnosis. Prognostic factors have been formulated in the past [4, 5, 8] but real progress has been made since Rai *et al.* [10] introduced a clinical staging system which was based on physical examination and simple laboratory data. The value of this approach has been confirmed by other authors [2, 9].

Recently the response to therapy as a prognostic factor has received more attention [2, 6, 7, 11]. We studied retrospectively

the response to therapy on survival in 56 patients with CLL.

### Patients and Methods

The diagnosis CLL (56 patients) was established when the lymphocytes in the peripheral blood were above  $10 \times 10^9/l$  and in the bone marrow over 40%. Clinical staging was performed as described by Rai *et al.* [10]. In short, stage 0, lymphocytosis; stage I, lymphocytosis plus enlarged lymph nodes; stage II, lymphocytosis plus enlarged liver or spleen; Stage III, lymphocytosis plus anemia; stage IV, lymphocytosis plus thrombocytopenia.

Survival was calculated from the time of diagnosis until death or up to May 1978. Actuarial survival curves were made according to Berkson and Gage [1]. Statistical analysis of the actuarial

Our studies thus indicate, that, as in various lymphoid malignancies, in CML also, the cells of the granulocytic series are agglutinated by Con A. Leucocytes from CML patients in remission from normal subjects, from patients with granulocytosis and normal immature myeloid cells from the bone marrow are not agglutinated by Con A. This alteration in the cell surface of the myeloid cells in CML patients occurs in immature cells as well as mature granulocytes.

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The differences in primary remission rates of chlorambucil, chlorambucil plus prednisone, CVP treatment and prednisone were significant ( $\chi^2 = 8.57$   $p = 0.04$ ), indicating less remissions for patients treated with prednisone and more remissions for patients treated with chlorambucil plus prednisone.

#### Results of Therapy

A complete remission was attained in 7 (14%) patients, a partial remission in 25 pa-

tients (49%). Improvement on therapy occurred in 15 patients (29%) and 4 patients (8%) failed therapy. Figure 1 presents the actuarial survival curves for complete and partial remission patients as well as improvement and failure patients over 10 years. Median survival for patients with complete or partial remission was 107 months. Patients who improved or failed therapy had a median survival of 26 months. The actuarial survival curves showed a significant difference (Breslow test,  $p < 0.001$ ).

The response to therapy related to clinical staging [10] is presented in table I. The remission rate for patients in stage 0+I+II was 66% (19 out of 29) and in stage III+IV 59% (13 out of 22). These differences were not significant ( $\chi^2 = 0.03$   $p = 0.86$ ). The 5-year survival fraction of patients in stage 0+I+II and III+IV were 59% (13 out of 22) and 27% (3 out of 11), respectively. These differences were not significant ( $\chi^2 = 2.97$   $p = 0.08$ ). The 5-year survival fraction of patients with complete and partial remission was 72% (13 out of 18), whereas that of patients with failure or improvement only was 20% (3 out of 15). These differences were significant ( $\chi^2 = 8.93$   $p = 0.003$ ).

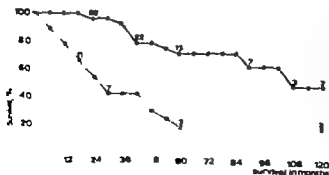


Fig. 1. The relation of survival with response to therapy. Actuarial survival curves of patients with complete remission and partial remission, compared to patients who either failed or only improved with therapy. The figures indicate the patients at risk for that period. O = Improvement and failure; ● = remission.

survival curves was performed as described by Breslow [3]. The 5-year survival fractions were determined by evaluating the patients (33) who were diagnosed before May 1973.

Survival was related to the best response to therapy. The result of therapy was defined as follows.

**Complete remission.** all of the following criteria. hemoglobin  $>77$  mmol/l, thrombocytes  $>150 \times 10^9/l$ , normal WBC with  $>50\%$  granulocytes, lymphocytes in the bone marrow  $<30\%$  of nucleated cells, spleen and liver not enlarged, no pathological lymph nodes, no symptoms of disease.

**Partial remission.** at least four of the following criteria. hemoglobin  $>67$  mmol/l, thrombocytes  $>100 \times 10^9/l$ , lymphocytes peripheral blood  $<15 \times 10^9/l$ , lymphocytes in the bone marrow  $<40\%$  of nucleated cells, spleen less than 3 cm below costal margin, lymph nodes less than 3 cm diameter no other symptoms of disease.

**Improvement.** favorable result of therapy on at least one of the above mentioned parameters.

**Failure.** unfavorable or no response to therapy

## Results

In the group of 56 patients were 37 men and 19 women. 30 patients were still alive at the time of evaluation in May 1978. Median survival for the whole group was 88 months. Therapy was given to 51 patients.

### Clinical Staging

The value of the clinical staging system according to Rai *et al.* [10] was confirmed, except for patients in stage IV. Median survival for patients in stage 0 (7 patients) was  $>132$  months, stage I (12 patients) 106 months, stage II (13 patients) 58 months, stage III (6 patients) 48 months and stage IV (18 patients) 51 months. Median survival for stage IV patients (14) in the study of Rai *et al.* [10] was 19 months.

When stage IV is restricted to patients who show the combination anemia plus

thrombocytopenia, as suggested by Phillips *et al.* [9] the median survival (11 patients) was 20 months.

### Modes of Therapy

**Chlorambucil.** Chlorambucil was given to 29 patients as primary treatment. This resulted in 10 partial remissions (34%). Chlorambucil was given to 5 patients as secondary treatment (3 after prednisone, 1 after cyclophosphamide, vincristine and prednisone (CVP), 1 after chlorambucil plus prednisone). This resulted in 2 additional partial remissions (after prednisone). The overall remission rate was 35% (12 out of 34).

**Chlorambucil plus Prednisone.** The combination chlorambucil and prednisone was given to 10 patients as primary treatment. 1 complete and 6 partial remissions (70%) were observed. In 9 patients this combination therapy was given as secondary treatment (8 after chlorambucil, 1 after prednisone). This resulted in 2 additional complete remissions (1 after chlorambucil, 1 after prednisone) and 3 partial remissions (after chlorambucil). The overall remission rate for chlorambucil plus prednisone was 63% (12 out of 19).

**CVP.** Combination therapy with CVP was given to 4 patients as primary treatment. This resulted in 1 complete and 2 partial remissions. In 12 patients it was given either as secondary treatment (9  $\times$  4 after chlorambucil, 5 after chlorambucil plus prednisone) or as tertiary treatment (3  $\times$  1 after chlorambucil, 2 after chlorambucil plus prednisone). This resulted in 2 additional complete remissions (1 after chlorambucil, 1 after chlorambucil plus prednisone, had already a partial remission) and 3 partial remissions (after chlorambucil). The overall remission rate was 50% (8 out of 16).

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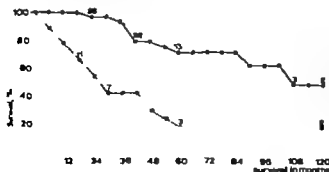


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Table 1. Relationship between clinical staging [10] and response to therapy

Stage	Number of patients with			
	complete remission	partial remission	improvement	failure
0	1	3	1	0
I	1	7	3	11
II	1	6	4	2
III	1	2	2	1
IV	3	7	5	1

In contrast to the findings of *Blinet et al* [2] patients in stage 0 and I do not have a greater chance for a remission than patients in stage IV

## Discussion

Clinical staging [*Rai et al* 10] in CLL is a useful tool to determine prognosis for individual patients. Our data confirmed the value of the staging system of *Rai et al* [10] with the exception of stage IV patients. Only when stage IV was restricted to patients with the combination of anemia plus thrombocytopenia a short median survival (20 months), comparable to the stage IV patients in *Rai et al* [10] (19 months) was observed. Also *Phillips et al* [9] suggested to classify patients with the combination anemia plus thrombocytopenia as stage IV. Besides clinical staging, response to therapy appeared to be an important factor in determining survival. Significantly different survival curves were obtained between patients who did or did not show a remission (fig. 1). In this study response to therapy was more valuable in determining 5-year survival compared to patients with 0+I+II disease or III+IV disease.

The differences in 5-year survival fractions for patients with stage 0+I+II (59%)

and III+IV (27%) were not significant. In contrast, the 5-year survival fractions of patients with a complete or partial remission (72%) compared to patients who showed improvement or failure (20%) were significantly different.

Other authors also described the correlation between prognosis and response to therapy in CLL [2, 6, 7, 11]. *Blinet et al* [2] found that clinical staging was related to the result of treatment. A larger number of patients who showed no response to therapy had a more advanced disease. As can be seen from table 1 we cannot confirm these findings. However the question whether response to therapy is more important than clinical stage in determining survival, is suggested in this study needs to be answered in a larger clinical investigation.

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Table I. Relationship between clinical staging [10] and response to therapy

Stage	Number of patients with			
	complete remission	partial remission	Improvement	failure
0	1	3	1	0
I	1	7	3	0
II	1	6	4	2
III	1	2	2	1
IV	3	7	5	1

In contrast to the findings of *Binet et al.* [2] patients in stage 0 and I do not have a greater chance for a remission than patients in stage IV

## Discussion

Clinical staging [*Rai et al.* 10] in CLL is a useful tool to determine prognosis for individual patients. Our data confirmed the value of the staging system of *Rai et al.* [10] with the exception of stage IV patients. Only when stage IV was restricted to patients with the combination of anemia plus thrombocytopenia, a short median survival (20 months) comparable to the stage IV patients in *Rai et al.* [10] (19 months) was observed. Also *Phillips et al.* [9] suggested to classify patients with the combination anemia plus thrombocytopenia as stage IV. Besides clinical staging, response to therapy appeared to be an important factor in determining survival. Significantly different survival curves were obtained between patients who did or did not show a remission (fig. 1). In this study response to therapy was more valuable in determining 5-year survival compared to patients with 0+I+II disease or III+IV disease.

The differences in 5 year survival fractions for patients with stage 0+I+II (59%)

and III+IV (27%) were not significant. In contrast, the 5-year survival fractions of patients with a complete or partial remission (72%) compared to patients who showed improvement or failure (20%) were significantly different.

Other authors also described the correlation between prognosis and response to therapy in CLL [2, 6, 7, 11]. *Binet et al.* [2] found that clinical staging was related to the result of treatment. A larger number of patients who showed no response to therapy had a more advanced disease. As can be seen from table I we cannot confirm these findings. However the question whether response to therapy is more important than clinical stage in determining survival, as suggested in this study needs to be answered in a larger clinical investigation.

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(American Instrument Co.), and the estimation of the level of 2,3-DPG in freshly collected blood samples (Sigma Co.). Structural identification of the unstable Hb variant was performed in Dr Halman's Protein Chemistry Laboratory. Over a period of 2 years, total of approximately 150 ml EDTA blood were collected on various clinic visits and sent to Augusta, Ga., packed in ice by air mail special delivery. 200 mg of the abnormal chain ( $\beta^x$ ) of the unstable haemoglobin was isolated by treatment with *p*-chloromercuribenzoate (PCMB; sodium salt, Nutritional Biochemical Corp., Cleveland, Ohio) [11], aminoethylated [15] and digested with trypsin. The resulting tryptic peptides were recovered by Chromobond F column chromatography [8] and analyzed with Beckman 121 M automatic amino acid analyzer. Family studies included haemolytic and haemoglobinopathic screening on both parents and siblings. True parentage was established by specific blood group typing.

## Results

Haematological data of the proband and other members of the family are presented in table I. The proband was anaemic with persistent reticulocytosis and elevated Heinz bodies. Red cell morphology showed fragments, target cells, basophilic stippling and Howell-Jolly bodies. Haemolytic studies showed increased bilirubins, the presence of free plasma haemoglobin and the depletion of plasma haptoglobins. Re-

sults were compatible with a haemolytic process.

Haemoglobin screening by starch gel electrophoresis at pH 9.0 did not reveal an abnormal haemoglobin except an extra minor haem-stained band in the position of the free  $\alpha$ -chain behind Hb A<sub>2</sub>. Both isopropanol and heat stability tests were indicative of the presence of 20–25% of an unstable haemoglobin variant (Hb X). The quantities of the various Hb fractions estimated by column chromatography were: Hb A + Hb X = 88.7%, Hb A<sub>2</sub> = 3.6%, free  $\alpha$ -chain = 1.0%, and Hb F = 6.7%. The functional studies showed a right shift of the oxygen dissociation curve of the whole blood with a  $P_{50}$  of 32 mm Hg (control 26 mm Hg). The 2,3-DPG level was elevated to 7.0 mmol/l RBC (normal range 2.3–6.7 mmol/l RBC).

Structural analysis of the tryptic peptides of the  $\beta^x$ -chain showed that all peptides had the expected amino acid composition except T 3 (amino acid residues 18–30 inclusive). Amino acid analysis of the abnormal T 3 gave the following data. Arg 0.84 (1), Asp 2.07 (2), Glu 2.14 (2), Pro 1.21 (0), Gly 2.98 (3), Ala 1.08 (1), Val 2.68 (3) and Leu 0.10 (1), (the numbers in parentheses refer to the expected numbers of residues in a normal T 3). The presence of an extra propyl residue and the absence of a

Table I. Haematological data of the patient and other family members

	Age years	Hb g%	RBC 10 <sup>6</sup> / $\mu$ l	MCV fl	MCH pg	MCHC %	Ratio %	Hb A <sub>2</sub> %	Hb F %	Isopropanol test	Free alpha chains	Heinz bodies %
Patient	5	10.5	4.08	83	5.8	30.7	28.0	3.6	6.7	+	+	9
Father	30	14.3	4.88	96	29.3	34.4	0.1	3.6	0.9	—	—	<0.5
Mother	28	12.6	4.24	87	29.7	34.2	0.1	3.9	1.2	—	—	<0.5
Brother	6	11.7	4.52	80	25.8	32.1	0.5	3.5	2.0	—	—	<0.5



## Short Communications

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### A New Case of the Unstable Haemoglobin Genova ( $\alpha_2\beta_2^{28}(\beta^{10})\text{Leu} \rightarrow \text{Pro}$ ) in Canada As a Result of Sporadic Mutation and Causing Heinz Body Haemolytic Anaemia

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**Key Words.** Haemolytic anaemia Hb Genova Sporadic mutation  
Unstable haemoglobins

**Abstract.** The first case of the unstable Hb Genova as a result of sporadic mutation is described. It is found in a 3-year-old Canadian boy of East Indian extraction with chronic Heinz body haemolytic anaemia.

Haemoglobin Genova ( $\beta^{28}\text{Leu} \rightarrow \text{Pro}$ ) is one of the more common unstable haemoglobins. It has been reported in four families of European descent, including one in Canada, and also in one Kenyan family in East Africa [9, 10, 12-14, 16]. This report describes a new case of Hb Genova in a Canadian boy of East Indian extraction as the result of a sporadic mutation and causing Heinz body haemolytic anaemia.

costal margin. There was no increase in jugular venous pressure nor peripheral oedema. Lung fields were clear. Height was stable on the 10th percentile and weight on the 75th percentile for his age over a 3-year period of observation. The patient was on long-term folic acid.

#### Materials and Methods

Standard laboratory procedures were used for haematological evaluations [5]. Haemolytic studies included the Heinz bodies preparation, quantitation of direct and indirect bilirubins, detection of free plasma haemoglobin and the semiquantitation of haptoglobins [2]. Haemoglobinopathy investigations involved starch gel electrophoresis [6], quantitation of Hb F by alkali denaturation [3], quantitation of free  $\alpha$ -chain and Hb A<sub>2</sub> by DE-52 column chromatography [1], and detection of unstable haemoglobin by isopropanol and heat stability tests [4, 7]. Functional studies included the determination of  $P_{50}$  values of whole blood at 37°C using a Hem-O-Scan oxygen dissociation analyzer

#### Case History

Our patient was a 3-year-old boy who was referred to the McMaster University Health Sciences Centre in Hamilton for the investigation of chronic anaemia. Physical examination revealed no pallor, slight scleral icterus and modest tachycardia with a mild haemic systolic murmur. The firm smooth liver edge was 6 cm below the right costal margin and the spleen 3 cm below the left

(American Instrument Co.), and the estimation of the level of 2,3-DPG in freshly collected blood samples (Sigma Co.). Structural identification of the unstable Hb variant was performed in Dr Hatowen's Protein Chemistry Laboratory. Over a period of 2 years, a total of approximately 130 ml EDTA blood were collected on various clinic visits and sent to Augusta, Ga., packed in ice by air mail special delivery 200 mg of the abnormal chain ( $\beta^s$ ) of the unstable haemoglobin was isolated by treatment with *p*-chloromercuribenzoate (PCMB<sup>-</sup> sodium salt, Nutritional Biochemical Corp., Cleveland, Ohio) [11], aminothylated [15] and digested with trypsin. The resulting tryptic peptides were recovered by Chromobead P column chromatography [11] and analyzed with Beckman 121 M automatic amino acid analyzer. Family studies included haemolytic and haemoglobinopathic screening on both parents and sibling. True parenthood was established by specific blood group typing.

## Results

Haematological data of the propositus and other members of the family are presented in table I. The propositus was anaemic with persistent reticulocytosis and elevated Heinz bodies. Red cell morphology showed fragments, target cells, basophilic stippling and Howell-Jolly bodies. Haemolytic studies showed increased bilirubins, the presence of free plasma haemoglobin and the depletion of plasma haptoglobins. Re-

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Table I. Haematological data of the patient and other family members

	Age years	Hb g%	RBC 10 <sup>12</sup> /μl	MCV fl	MCH pg	MCHC %	Retic %	Hb A %	Hb F %	Isopropanol test	Free alpha chains	Heinz bodies %
Patient	5	10.5	4.08	83	25.8	30.7	28.0	3.6	6.7	+	+	9
Father	30	14.3	4.88	86	29.3	34.4	0.1	3.6	0.9	-	-	<0.5
Mother	28	12.6	4.4	87	29.7	34.2	0.1	3.9	1.1	-	-	<0.5
Brother	6	11.7	4.52	80	25.8	32.1	0.5	3.5	1.0	-	-	<0.5

Table II. Blood group studies

*Blood group typing*

Patient  $\text{Cde/Cde}$ , M+ N+ S+  $\overline{\text{I}}$   $\overline{\text{kk}}$ , C<sup>-</sup> negative,  
 Jk (a+b-), Fy (a-b+)  
 Father  $\text{Cde/Cde}$ , M- N+ S-  $\overline{\text{I}}$   $\overline{\text{kk}}$ , C<sup>-</sup> negative,  
 Jk (a+b-) Fy (a-b+)  
 Mother  $\text{CDe/Cde}$ , M+ N+ S+  $\overline{\text{I}}$   $\overline{\text{kk}}$ , C<sup>-</sup> negative,  
 Jk (a-b+) Fy (a-b+)

*Secretor genotype from Lewis typing*

Patient Se, Se  
 Father Se, Se  
 Mother Se, Se

*HLA typing*

Patient A<sub>2</sub>, B<sub>27</sub> B<sub>17</sub> CW<sub>2</sub>  
 Father A<sub>2</sub>, A<sub>2</sub>, B<sub>27</sub> B<sub>17</sub> CW<sub>2</sub>  
 Mother A<sub>2</sub>, B<sub>17</sub> B<sub>27</sub> CW<sub>2</sub>, CW<sub>3</sub>

These data do not prove paternity. They simply do not exclude it.

leucyl residue in the T 3 indicated a Leu → Pro replacement at position 28, as found in Hb Genova.

Blood group studies including RBC genotyping, secretor status and HLA typing (table II) established that the parents were the biological parents of the propositus. Haemolytic and haemoglobinopathic screening on both parents and the older brother of the propositus revealed no abnormality indicating that the presence of the unstable Hb Genova in the propositus was the result of a fresh mutation and responsible for the clinical haemolysis.

## Discussion

Since its initial discovery in an Italian family in 1965 and its subsequent occurrence in five other families [9 10 12-14] Hb Genova has been found only in one

Canadian woman of Ukrainian-Scottish-German extraction [16] who probably inherited the unstable haemoglobin variant from her Ukrainian father. This report described the second case of Hb Genova in Canada found in an East Indian boy. Genotyping and family studies indicated that the unstable haemoglobin in our patient was due to a sporadic mutation. The presence of approximately 25% of an unstable haemoglobin in our patient resulted in clinical manifestations similar to previously reported cases, namely chronic haemolytic anaemia with persistent reticulocytosis and elevated Heinz bodies. This case of an unstable haemoglobin due to a fresh mutation illustrates the importance of family studies in the investigation of haemoglobinopathies and the usefulness of genotyping to establish parenthood.

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## Effect of Polyamines on Autohemolysis Studies on Normal and Thalassemic Children

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**Key Words:** Hemolysis Polyamines Superoxide dismutase  $\beta$ -Thalassemia

**Abstract.** The effect of glucose and polyamines (putrescine, spermidine and spermine) on the rate of autohemolysis in normal and thalassemic red blood cell (RBC) populations has been investigated. In addition, the specific activity of superoxide dismutase (SOD) was measured on blood samples obtained from the same patients. Our results show that glucose, and to a less extent polyamines, reduce the autohemolysis of thalassemic RBC, and that specific activity of SOD is increased in RBC from thalassemic patients compared to normal subjects.

Auto-oxidation of biomembranes is considered to be the primary factor involved in cellular senescence and breakdown [1, 2]. An increased production of highly activated forms of oxygen released during the oxidation of hemoglobin to methemoglobin in thalassemic red blood cells (RBC) [3-8] has stimulated much interest in superoxide dismutase (SOD) and cellular antioxidants for the control of such deleterious radical reactions.

In the past years, the role of polyamines as cellular antioxidants and membrane stabilizers has been suggested [9]. Recently it has been shown that polyamines can scavenge superoxide anion generated *in vitro* by the hypoxanthine-xanthine oxidase system [10]. In the present paper we report the

effect of polyamines (putrescine, spermidine and spermine) on autohemolysis of erythrocytes obtained from homozygotic  $\beta$ -thalassemic patients.

### Materials and Methods

#### *Autohemolysis*

Erythrocytes were obtained from 25 homozygotic  $\beta$ -thalassemic patients aged 3-10 years. These patients had received no blood transfusion for approximately 25 days. Hemoglobin levels in these patients ranged from 6 to 8.5 g/dl.

Red cells of normal subjects of similar age were used as control. Autohemolysis was determined according to the method of Selwyn and Dacie [11]: sterile and defibrinated blood samples (2 ml) were transferred into glass tubes and placed for 48 h at 37 °C.

Polyamines (2.5 mM) or glucose (60 mM) were added to aliquots of erythrocytes prior to the incubation. After incubation, hemoglobin concentration was determined spectrophotometrically at 545 nm.

#### Superoxide Dismutase Assay

Heparin-anticoagulated blood was obtained from the same patients. The specific activity of SOD was assayed in chloroform-ethanol extracts of the hemolysates according to the method of *M. Card and Fridovich* [12]. RBC were washed 3 times with cold physiological saline and lysed by adding 2 vol of cold water. Hemoglobin was precipitated after 2 h of incubation at 4 °C, with 11 ml of chloroform-ethanol mixture (3:5, v/v) at 0 °C, followed by adding 0.5 ml of water. The suspension was centrifuged for 10 min at 3,000 g and the pale yellow supernatant was used for estimating the specific activity of SOD, which is reported as percent inhibition of the reduction of cytochrome c per milligram of proteins. Under these conditions, the amount of SOD capable to inhibit reduction of cytochrome c by 50% at 25 °C was defined as 1 enzyme unit (EU) of activity.

The superoxide anion was routinely generated *in vitro* with the xanthine oxidase-hypoxanthine system according to the method of *Fridovich* [13]. The reaction mixture contained in total volume of 1 ml: 100 µmol glycyl-glycine buffer (pH 7.8), 1.5 µmol hypoxanthine, 0.1 mg xanthine oxidase (spec. act. 0.4 U/mg), 3.5 µmol cytochrome c; the reaction was initiated with chloroform-ethanol extract containing approximately 2–4 µg/ml of proteins. Proteins were determined by the method of *Lewry et al.* [14].

#### Results

Figure 1 shows the effect of polyamines or glucose on autohemolysis of erythrocytes obtained from thalassaemic patients and normal subjects, respectively. As can be seen, the addition of polyamines reduces the autohemolysis in an abnormal erythrocyte population. Putrescine, spermidine and spermine have no effect on autohemolysis of normal RBC. At elevated levels of po-

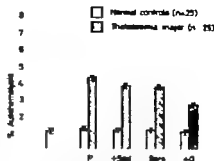


Fig. 1. Effect of polyamines and glucose on autohemolysis of erythrocytes obtained from normal and thalassaemic subjects (mean values  $\pm$  1 SD). N. a. = No addition, P = putrescine 2.5 mM, Spd = spermidine 2.5 mM, Spm = spermine 2.5 mM, G = glucose 60 mM.

lyamines, no significant change in the rate of hemolysis was found in thalassaemic RBC. Furthermore, a lower rate of autohemolysis is observed when glucose, rather than polyamines, is added to thalassaemic RBC. In 2 other thalassaemic children an attempt was made to evaluate whether the protective effects of glucose and polyamines were additive. In the 1st patient the autohemolysis rate decreased to 18% of the basal value with glucose, to 30% with spermine, and to 12% with glucose + spermine. In the 2nd patient the respective values were 41, 62, and 39%. The effects of the two substances being at least partially additive, this would suggest that the underlying mechanisms are dissimilar.

In view of reports in the literature [15] suggesting that SOD is not elevated in thalassaemic RBC, we have compared the levels of SOD in normal and thalassaemic individuals. The specific activity of SOD was significantly increased in thalassaemic RBC as compared to normal subjects [ $360 \pm 10$  and  $170 \pm 8$  U/mg protein, respectively

## Effect of Polyamines on Autohemolysis: Studies on Normal and Thalassemic Children

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**Key Words:** Hemolysis · Polyamines · Superoxide dismutase ·  $\beta$ -Thalassemia

**Abstract.** The effect of glucose and polyamines (putrescine, spermidine and spermine) on the rate of autohemolysis in normal and thalassemic red blood cell (RBC) populations has been investigated. In addition, the specific activity of superoxide dismutase (SOD) was measured on blood samples obtained from the same patients. Our results show that glucose, and to a less extent polyamines, reduce the autohemolysis of thalassemic RBC, and that specific activity of SOD is increased in RBC from thalassemic patients compared to normal subjects.

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#### Superoxide Dismutase Assay

Heparin-anticoagulated blood was obtained from the same patients. The specific activity of SOD was assayed in chloroform-ethanol extracts of the hemolysates according to the method of *M. Cord and Fridlovich* [12]. RBC were washed 3 times with cold physiological saline and lysed by adding 2 vol of cold water. Hemoglobin was precipitated after 2 h of incubation at 4°C, with 0.8 ml of chloroform-ethanol mixture (3.5, v/v) at 0°C, followed by adding 0.8 ml of water. The suspension was centrifuged for 10 min at 3,000 g, and the pale yellow supernatant was used for estimating the specific activity of SOD which is reported as percent inhibition of the reduction of cytochrome c per milligram of protein. Under these conditions, the amount of SOD capable to inhibit reduction of cytochrome c by 50% at 25°C was defined as 1 enzyme unit (EU) of activity.

The superoxide anion was routinely generated *in vivo* with the xanthine oxidase-hypoxanthine system according to the method of *Fridlovich* [13]. The reaction mixture contained in total volume of 1 ml, 100  $\mu$ mol glycyl-glycine buffer (pH 7.8), 1.5  $\mu$ mol hypoxanthine, 0.1 mg xanthine oxidase (spec. act. 0.4 U/mg), 3.5  $\mu$ mol cytochrome c; the reaction was initiated with chloroform-ethanol extract containing approximately 2-4  $\mu$ g/ml of proteins. Proteins were determined by the method of *Lovry et al.* [14].

#### Results

Figure 1 shows the effect of polyamines or glucose on autohemolysis of erythrocytes obtained from thalassaemic patients and normal subjects, respectively. As can be seen, the addition of polyamines reduces the autohemolysis in an abnormal erythrocyte population. Putrescine, spermidine and spermine have no effect on autohemolysis of normal RBC. At elevated levels of po-

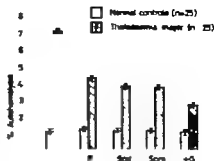


Fig. 1. Effect of polyamines and glucose on autohemolysis of erythrocytes obtained from normal and thalassaemic subjects (mean values  $\pm$  1 SD). N. a. = N addition; P = putrescine 2.5 mM Spd = spermidine 2.5 mM Spm = spermine 2.5 mM G = glucose 60 mM

lyamines, no significant change in the rate of hemolysis was found in thalassaemic RBC. Furthermore, a lower rate of autohemolysis is observed when glucose, rather than polyamines, is added to thalassaemic RBC. In 2 other thalassaemic children an attempt was made to evaluate whether the protective effects of glucose and polyamines were additive. In the 1st patient the autohemolysis rate decreased to 18% of the basal value with glucose, to 30% with spermine, and to 12% with glucose + spermine. In the 2nd patient the respective values were 41, 62, and 39%. The effects of the two substances being at least partially additive, this would suggest that the underlying mechanisms are dissimilar.

In view of reports in the literature [15] suggesting that SOD is not elevated in thalassaemic RBC, we have compared the levels of SOD in normal and thalassaemic individuals. The specific activity of SOD was significantly increased in thalassaemic RBC as compared to normal subjects [ $360 \pm 10$  and  $170 \pm 8$  U/mg protein, respectively



## Effect of Polyamines on Autohemolysis Studies on Normal and Thalassaemic Children

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**Key Words.** Hemolysis Polyamines Superoxide dismutase  $\beta$ -Thalassaemia

**Abstract.** The effect of glucose and polyamines (putrescine, spermidine and spermine) on the rate of autohemolysis in normal and thalassaemic red blood cell (RBC) populations has been investigated. In addition, the specific activity of superoxide dismutase (SOD) was measured on blood samples obtained from the same patients. Our results show that glucose, and to a less extent polyamines, reduce the autohemolysis of thalassaemic RBC, and that specific activity of SOD is increased in RBC from thalassaemic patients compared to normal subjects.

Auto-oxidation of biomembranes is considered to be the primary factor involved in cellular senescence and breakdown [1-2]. An increased production of highly activated forms of oxygen released during the oxidation of hemoglobin to methemoglobin in thalassaemic red blood cells (RBC) [3-8] has stimulated much interest in superoxide dismutase (SOD) and cellular antioxidants for the control of such deleterious radical reactions.

In the past years, the role of polyamines as cellular antioxidants and membrane stabilizers has been suggested [9]. Recently it has been shown that polyamines can scavenge superoxide anion generated *in vitro* by the hypoxanthine-xanthine oxidase system [10]. In the present paper we report the

effect of polyamines (putrescine, spermidine and spermine) on autohemolysis of erythrocytes obtained from homozygotic  $\beta$ -thalassaemic patients.

### Materials and Methods

#### *Autohemolysis*

Erythrocytes were obtained from 25 homozygotic  $\beta$ -thalassaemic patients aged 3-10 years. These patients had received no blood transfusion for approximately 25 days. Hemoglobin levels in these patients ranged from 6 to 8.5 g/dl.

Red cells of normal subjects of similar age were used as control. Autohemolysis was determined according to the method of Selwyn and Dacie [11]. Sterile and defibrinated blood samples (2 ml) were transferred into glass tubes and placed for 48 h at 37 °C.

and polyamines might be more effective in protecting biomembranes against the deleterious effect of free radicals.

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(mean  $\pm$  SD values)  $p < 0.001$  by Student's  $t$  test]

## Discussion

The thalassemic RBC offer an interesting model system for studying degenerative processes of cellular membranes due to auto-oxidation reactions. In  $\beta$ -thalassemic RBC, increased precipitation of  $\alpha$ -chains associated with increased oxidation of heme iron has been cited as the principal mechanism for generating activated species of molecular oxygen [3-8]. Conceivably these activated forms of oxygen can be formed during transport of oxygen through the membrane as well as within the cell. In this context, the role of small molecules endowed with antioxidant activity would be more effective in protecting membrane lipids from undergoing free radical chain reactions. In contrast, SOD being effective in the dismutation of superoxide anion in the cell, may be not accessible to the lipid matrix of the membrane due to its size and charged groups. Consequently the effective radical scavenging action of SOD within the membrane would be markedly diminished.

The evidence reported in this communication demonstrates that both glucose and polyamines can reduce thalassemic RBC hemolysis *in vitro* probably acting by different mechanisms.

The protective effect of glucose is more pronounced than the effect of polyamines. According to *Malzels* [16] it probably exerts its effect by providing energy for the synthesis of organic phosphate compounds, particularly adenosine triphosphate (ATP) and diphosphoglycerate. Furthermore, it might increase the concentration of reduced

glutathione by a greater availability of substrate in the pathway shunt [17].

While at the present time the mechanism by which polyamines retard hemolysis is not clear it is tempting to speculate on their effective role as free radical scavengers and membrane stabilizers. Our observations that polyamines, particularly spermine, can inhibit cytochrome *c* reduction by the superoxide anion suggest that these nitrogenous bases can interact with the radical anion of oxygen [10]. The membrane stabilization by polyamines could be a function of their size and polycationic properties which could favor hydrophobic interactions within the membrane and thus retard hemolysis. These observations are in agreement with those obtained by *Tabor and Tabor* [18] who showed that oxidation of unsaturated fatty acids is inhibited by spermine.

Measurements of the specific activity of SOD in thalassemic RBC show a higher percent inhibition of cytochrome *c* per milligram of protein as compared with normal individuals. The increase of SOD specific activity found in RBC of thalassemic patients, may be associated with increased production of superoxide anion. An induction of SOD was demonstrated in experiments on oxygen toxicity [19-21].

Our results concerning the increased specific activity of SOD in thalassemic RBC do not agree with reports from other laboratories [15] which have observed no differences in the level of SOD in normal and thalassemic RBC. Conceivably the different assay methods employed in such comparison might explain the discrepancy. The interesting observation that emerges from this work is that high SOD activities found in thalassemic RBC apparently do not protect them from increased rate of autohemolysis,

and polyamines might be more effective in protecting biomembranes against the deleterious effect of free radicals.

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## Autoimmune Antibodies after Splenectomy

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**Key Words.** Splenectomy Autoimmune Antibodies Suppressor T cell

**Abstract.** Sera of 41 splenectomized subjects were tested for presence of autoantibodies by the indirect immunofluorescent method. 11 of them (26%) revealed the presence of one or more types of autoantibodies. Antiheart antibodies were the most common type (9 subjects). The incidence of antibodies was higher in the subjects splenectomized for trauma than in those who underwent elective splenectomy (39 and 25%, respectively). There was no significant difference between the subjects with positive and negative results with respect to mean age at examination, but the positive results tend to occur more frequently in those who underwent splenectomy at younger age. None of the subjects with any type of antibodies suffered from autoimmune disease.

Although it is still impossible to evaluate the clinical importance of these findings, they suggest that splenectomy may disturb the regulatory processes in the immune system. We postulate that this is due to the reduction of the number of suppressor T cells for which the spleen is a major pool.

### Introduction

The first suggestion of a possible connection between the spleen and autoimmunity was made by Wardrop *et al* [1975] who described a high incidence of autoimmune antibodies in 14 elderly patients with splenic atrophy. These authors could not exclude, however, the possibility that the splenic atrophy was the result rather than the cause of an autoimmune disease.

Since the major limitation of this type of study is the lack of laboratory information

on the patients' immunological state prior to the appearance of splenic atrophy, we decided to investigate the presence of autoantibodies in patients who underwent splenectomy for various reasons in our institution.

### Material and Methods

41 patients (27 males and 14 females) who underwent splenectomy in the Municipal-Governmental Medical Center Tel Aviv-Jaffa, during the last 20 years were included in this study. 33

splenectomized for traumatic rupture of the spleen and 8 underwent elective splenectomy (spherocytosis, ITP, Gaucher's disease). At the time of examination, their age ranged from 5 to 39 years. They were splenectomized 2 months to 20 years before the study was performed.

Two control groups were chosen. One group consisted of 10 normal healthy volunteers from medical and nursing staff, matched for sex and age. The second group consisted of age- and sex-matched patients who underwent abdominal surgery for nonmalignant disease.

From each subject, venous blood was obtained and serum stored at  $-20^{\circ}\text{C}$  prior to examination. The following tests were performed: direct and indirect Coombs test, antinuclear (ANA), antimitochondrial (AMA), antismooth muscle (ASMA), antiparietal cells (APA), and antihistamine (AHA) antibodies.

The presence of autoantibodies was tested by means of the indirect immunofluorescent method. Rat kidney sections were used for the detection of the mitochondrial and smooth-muscle antibodies (smooth muscle fibers in vessel walls).

## Results

Of 41 patients examined for the presence of autoantibodies, 11 (26%) manifested the presence of one or more types of autoantibodies. None of the subjects in either control group (healthy and those who underwent abdominal surgery) had positive results. The incidence of autoantibodies was higher in the subjects splenectomized for trauma than in those who underwent elective splenectomy (39 and 25%, respectively).

Table I summarizes the personal and laboratory findings of the 11 splenectomized patients in whom autoantibodies of various types were found. As can be seen, heart antibodies were the most common type of antibodies found (9 patients). 1 patient had antismooth muscle and one anti-

Table I. Personal and laboratory findings of the 11 subjects with antibodies

Subject No.	Age at transplantation years	Age at splenectomy years	Reason for splenectomy	Antibodies (titer)		AMA	AHA	ANA	RF	Coombs direct	Coombs indirect
				APA	AMA	IgG IgM					
1	32	19	trauma	N	N	W N	1 10	N	N	N	N
2	43	35	trauma	N	N	N N	1 10	N	N	N	N
3	25	15	trauma	N	N	N N	1 20	N	N	N	N
4	36	23	trauma	N	N	N N	1 20	N	N	N	N
5	21	19	trauma	N	N	N N	1 20	N	N	N	N
6	11	6	trauma	N	N	1 40 N	N	N	N	N	N
7	16	13	trauma	1:40	N	N N	1 40	N	N	N	N
8	37	33	trauma	1:20	N	N N	1 10	N	N	N	N
9	26	6	trauma	1 20	N	N N	N	N	N	N	N
10	6	5.5	ITP	N	N	N N	1 10	N	N	N	N
11	18	15	ITP	N	N	N N	1 10	N	N	N	N

ITP = idiopathic thrombocytopenic purpura; N = negative; APA = antiparietal cells antibodies; AMA = antimitochondrial antibodies; ASMA = antismooth muscle antibodies; AHA = antihistamine antibodies; ANA = antinuclear antibodies; RF = rheumatoid factor.

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5	21	19	trauma	N	N	N	N	1:20	N	N	N	N
6	11	6	trauma	N	N	1:40	N	N	N	N	N	N
7	16	13	trauma	1:40	N	N	N	1:40	N	N	N	N
8	27	23	trauma	1:20	N	N	N	1:10	N	N	N	N
9	26	6	trauma	1:20	N	N	N	N	N	N	N	N
10	6	5.5	ITP	N	N	N	N	1:10	N	N	N	N
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parietal cells antibodies alone. In 2 patients, two types of antibodies (AHA and APA) were found present simultaneously.

There was no significant difference between the patients with positive and negative results with respect to mean age at date of examination (23.7 vs. 25.6 years) and mean age at date of splenectomy (16.3 vs. 19.9 years). However, checking the results according to the age distribution at time of splenectomy, the positive results tend to occur more frequently in those subjects who underwent splenectomy at younger age.

## Discussion

In this study we presented a group of subjects without known immunological diseases, without tendency to streptococcal infections, without malabsorption syndrome and whose history includes only the important event of splenectomy. In over 25% of these patients, we detected a significant titer of at least one autoantibody. As the incidence of the antibodies was higher in the traumatic splenectomy than in the elective splenectomy group, we can conclude that our findings are not due to any basic disorder but to a possible direct consequence of splenectomy.

The incidence of the autoimmune phenomena is primarily affected by the age at which splenectomy was performed and bears no relation to the age of the patient when investigated nor to the time elapsed since splenectomy.

Although the significance of antihart autoantibodies in many clinical situations is still debated, it is well accepted that their prevalence in general population is about 1%, and, even after myocardial infarction does not exceed 3% [Golan 1977]. Thus,

25% prevalence in our splenectomized patients is significant and noteworthy.

The appearance of autoimmune antibodies in the postsplenectomy state may be an evidence of impaired regulatory function in the immune system. The regulatory effect of T lymphocytes on antibody production is now a widely accepted fact. The controlling action of T lymphocytes is via the two important subpopulations: the helper and the suppressor cells [Katz, 1977].

Recently based on animal models [Allison *et al.*, 1971; Steinberg *et al.*, 1975] it was suggested that loss of suppressor T cells or diminished suppressor T cell activity can lead to autoimmune disease. The possibility that the same events can lead to autoimmune disease found in man was also raised [Waldman and Broder, 1977].

Suppressor T cells can be found in the spleen [Sampson, 1975] and thymus but not in lymph nodes or peripheral blood [Sampson *et al.*, 1976, 1977]. The role of the spleen as a major source of suppressor T lymphocytes was recently established [Sampson, 1975; Asherson and Zembala, 1976] but the consequence of splenectomy on the aspect of autoimmune phenomena production has not yet been investigated.

Since, in our study we dealt with healthy subjects who underwent splenectomy mainly for traumatic rupture of the spleen, it may be speculated that the occurrence of autoantibodies is direct consequence of splenectomy, most probably by diminishing the number of suppressor T lymphocytes, cells for which the spleen is an important source and pool.

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parietal cells antibodies alone. In 2 patients two types of antibodies (AIIA and APA) were found present simultaneously.

There was no significant difference between the patients with positive and negative results with respect to mean age at date of examination (23.7 vs. 25.6 years) and mean age at date of splenectomy (16.3 vs. 19.9 years). However, checking the results according to the age distribution at time of splenectomy, the positive results tend to occur more frequently in those subjects who underwent splenectomy at younger age.

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Letter to the Editor

## Cryopathic Haemolytic Anaemia Associated with Uterus Myomatosis

Cold agglutinins are commonly found in patients with lymphoreticular neoplasms, and in some infectious and autoimmune disorders [2, 3]. Recently we have encountered patient with uterus myomatosis who developed cold-agglutinin-induced haemolytic anaemia. Hysterectomy was followed by the prompt disappearance of cold-reacting antibodies. The association of cryopathic haemolytic anaemia with non-lymphoreticular neoplasms is rare, and has not yet been described in uterus myomatosis.

### Case Report

A 38-year-old woman was admitted in May 1978 for evaluation of progressive weakness and anaemia. The spleen was palpated 6 cm below the costal margin and the uterus was grossly enlarged. The haemoglobin was 6 gm/dl, haematocrit 21% and reticulocytes 3%. Leucocytes and platelet counts were normal. Tests for antinuclear factor, LE cells, rheumatoid factor, mycoplasma and infectious mononucleosis were negative. Serum complement was 64 mg/dl (normal > 80 mg/dl). The direct Coombs' test was positive for  $C_{3d}$  and  $C$  and negative for IgG. The cold agglutinin titre was 1/2048 with IgM, anti-I specificity. Bone marrow aspiration revealed erythroid hyperplasia. The  $T_{10}$  red cell  $^{51}Cr$  survival was 8 days. On June 13th, 1978, hysterectomy was performed. A large uterine tumour was found, and histological examination was compatible with benign myoma. 10 days following hysterectomy the cold agglutinin titre declined to 1/32, the reticulocytes were 0.9% and the haemoglobin 12 g/dl. 1 year after surgery the patient was in perfect health without splenic enlargement. The haemoglobin was 15 g/dl, and cold agglutinins were undetectable.

### Discussion

A cause and effect relationship between the uterine tumour and the haemolytic anaemia is suggested by the prompt disappearance of cold agglutinins and cure of haemolytic anaemia following hysterectomy. Haemagglutinins have been shown to exist in some malignant tumours [4], but extracts of the tumour in the present case showed no haemagglutinating activity. Alternatively tumour-associated antigens may have stimulated the production of antibodies cross-reacting with the I antigen of autologous erythrocytes [1].

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# Correspondence

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## To the Editor

Professor Bernstein adds hemoglobin C  $Gd^4$  and  $\beta^S$ -globin to the list of African genes detected in contemporary Sicilian populations. He also points out the less specific role for cDe ( $Rh_o$ ) with which there is no disagreement.

The objective of our study [1] was to focus attention on the very high portion of Sicilian emigrants represented by case reports of white persons with sickle cell hemoglobin and, through a limited field survey to demonstrate that hemoglobin S was only one of several African genes readily identifiable in contemporary Sicilian populations by simple blood-typing techniques. We are reluctant to speculate from our limited data, as well as from data of other studies in the literature,

on the quantitation of African admixture in the contemporary Sicilian genetic constitution.

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# KargerGazette

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**Indications:** RhOGAM (Rh<sub>0</sub>(D) Immune Globulin (Human)) used to prevent the formation of active antibodies in an Rh<sub>0</sub>(D) negative, D<sup>+</sup> negative individual into whose bloodstream Rh<sub>0</sub>(D) positive or D<sup>+</sup> positive blood has entered. Such an occurrence might result from full term or early termination of an Rh incompatible pregnancy or from an Rh incompatible transfusion.

**Dosage:** One vial of RhOGAM will completely suppress sensitivity to 15 ml of Rh positive red blood cells (packed cells, not whole blood) in deciding the dose of RhOGAM which is to be administered to an individual, the volume of Rh positive blood which entered the person's bloodstream to be determined from such the dose of RhOGAM can be calculated. 1. The usual full-term delivery single dose four vial of RhOGAM will be adequate to suppress sensitivity to the Rh<sub>0</sub>(D) antigen. However in the case of large fetomaternal hemorrhage or an Rh incompatible transfusion accident, more than one dose may be indicated.

**Product Description:** RhOGAM (Rh<sub>0</sub>(D) Immune Globulin (Human)) is sterile concentrated solution of specific

immunoglobulin (IgG) containing anti Rh<sub>0</sub>(D). This product prepared from fractionated human plasma (cold alcohol method) and may include immunoglobulin, which does not contain anti-Rh<sub>0</sub>(D), derived from an intermediate plasma fraction prepared by other Branch of Biological Products manufacturers. **Action:** With an injection of passive Rh<sub>0</sub>(D) antibody (RhOGAM) to the postpartum mother postmiscarriage/abortion women, or to the recipient of transfusion accident, the passive antibody response to the foreign Rh<sub>0</sub>(D) positive cells suppressed.

**Caution:** Reactions of Rh<sub>0</sub>(D) negative individuals given Rh<sub>0</sub>(D) Immune Globulin (Human) are infrequent, of mild nature and mostly confined to the area of injection. An occasional patient may react more strongly both locally and generally. A slight elevation of temperature has been noted in small number of postpartum women.

Fever, myalgia, lethargy, increased fibrinolytic levels and/or oligomenorrhea have been infrequently observed in individuals following multi-dose regimen after Rh mismatched transfusion.

Systemic reactions are rare and sensitization due to repeated exposure of immune globulins is unusual.

Immune Serum Globulin (Human) has not been reported to transmit hepatitis.

RhOGAM is to be given to the recipient in transfusion accident or the postpartum mother or postmiscarriage women, only. It need not be given to the infant.

**Contraindications:** Rh<sub>0</sub>(D) Immune Globulin (Human) should not be administered to:

1. An Rh<sub>0</sub>(D) positive or D<sup>+</sup> positive individual.
2. An Rh<sub>0</sub>(D) negative patient who has inadvertently received an Rh<sub>0</sub>(D) positive blood transfusion within three months of delivery.
3. A patient previously sensitized to the Rh<sub>0</sub>(D) blood factor.

**Supplied:** In package containing single-dose vial of RhOGAM and package containing 25 single-dose vials of RhOGAM.

**NOTE:** For complete prescribing information, see directions circular.

# Rho

Rh<sub>0</sub>(D) Immune



## Book Review

F. Heckner

Praktikum der mikroskopischen Hämatologie  
4. Aufl. Urban & Schwarzenberg, München 1978  
XI + 121 pp. 105 fig. In 204 ein- und  
mehrfarbigen Teilbildern DM 36-  
ISBN 3-541-01004-5

Nebst den vielen hämatologischen Büchern nimmt auch die Anzahl der morphologischen Werke in letzter Zeit wieder zu. Man muss sich deshalb fragen, wo der Platz dieses Praktikums der mikroskopischen Hämatologie von Fritz Heckner ist. Die Erkennung der Blutkrankheiten findet, wie der Autor im Vorwort selber schreibt, in der ärztlichen Sprechstunde, am Krankenbett und im Laboratorium statt. Die fortgeschrittene Mechanisierung der Medizin hat jedoch bisher die richtige Deutung und exakte Unterscheidung von Blut und Knochenmarkszellen durch das Auge des Untersuchers nicht verdrängt. Das vorliegende Werk ist eine Sammlung von Bildern verschiedenster normaler und pathologischer Zellen von Blutbild und Knochenmark. Es ist deshalb als «morphologische Stütze» gut geeignet für das Routinelabor vor allem kleinerer Spitäler und größerer Praxen, wo seltene Krankheitsbilder nicht jeden Tag diagnostiziert werden. Die Einteilung des Buches ist übersichtlich, der Untersucher wird die gesuchten Zellen rasch finden. Die Erklärung der Bilder ist einfach, klar und stichhaltig. Am Schluss des Bu-

ches findet sich eine kurze Übersicht der wichtigsten Färbetechniken. Obwohl der Autor erklärt, dass er sich den neuesten Gegebenheiten angepasst hat, ist die Nomenklatur teilweise etwas veraltet. So werden beispielsweise klassische Echinozyten als Akanthozyten bezeichnet (p. 42), und recht häufig findet man den veralteten Ausdruck «Paramyelo- oder Paralympoblasten». Ferner fehlt die neue internationale Einteilung der Leukämie. Es ist ausserdem schade, dass zum Teil Schwarzweissbilder verwendet wurden, wo Farbabbildungen mehr aussagen würden. Etwas unglücklich und meines Erachtens auch überflüssig ist die Anleitung zur Befunderhebung von Knochenmarkspunktionen. Der Autor versucht hier aufgrund symbolisch dargestellter Zellen einen Überblick über die Knochenmarksbefunde der einzelnen Erkrankungen zu geben. Bis man sich jedoch diese Symbole gemerkt hat, ist man durch Konsultation der Farbabbildungen einfacher und schneller zum Ziel gekommen. Diese sind nämlich meist sehr scharf, deutlich und gut in der Farbe.

Trotz der geringen Einschränkungen ist dieses Praktikum der mikroskopischen Hämatologie, welches nur Hinweise auf die morphologische Diagnostik, nicht aber auf die zugrundeliegenden Krankheitsbilder geben will, für den praktisch tätigen Arzt und das Labor sehr geeignet.

G. F. Riedler Luzern

## Announcement

The American Red Cross Twelfth Annual Scientific Symposium entitled 'The Lymphocyte' will be held May 6-7 1980, in Washington, DC, at the Pan American Health Organization, 525 32nd Street, NW

All attendees must be preregistered. For further information please contact Ms. Betty Galab Symposium Coordinator American Red Cross Blood Services Laboratories, 9312 Old Georgetown Road, Bethesda, MD 20014 telephone: (301) 530-6040.

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**KEYWORDS:** *child abuse; child sexual abuse; child sexual exploitation; child sexual abuse investigation; child sexual abuse investigation team; child sexual abuse investigation unit; child sexual abuse investigation team; child sexual abuse investigation unit; child sexual abuse investigation team; child sexual abuse investigation unit*

Indicates Rh(GAM) Rh(D) positive (Globulin Clonate) used to prevent the formation of acute antibodies, as an Rh(D) negative, D<sup>+</sup> negative individual into whose bloodstream Rh(D) positive or D<sup>+</sup> positive blood has entered. Such an occurrence might result from the same or early transfusions of an Rh incompatible pregnancy or from an Rh incompatible transfusion.

**Dosage:** One vial of RhioGAM will completely suppress immunity to 15 ml of Rh positive red blood cells (nucleated cells, not just blood). In dosing the dose of RhioGAM back to be administered to an individual, the volume of Rh positive blood which entered the person's bloodstream is to be determined. From which the dose of RhioGAM can be calculated. In the usual full-term delivery, single dose (one vial) of RhioGAM will be adequate to suppress immunity to the Rh-DM antigens. However, in the case of large fetal-maternal hemorrhages or an Rh incompatible transfusion accident, more than one dose may be indicated.

**Product Description:** RhoGAM (Rh<sub>0</sub>(D)  
Immune Globulin (Human) sterile  
concentrated solution of specific

immunoglobulin (IgG) containing anti-Rh<sub>123</sub>. This product, prepared from fractionated human plasma (cold alcohol method) and may include immunoglobulins, both does not contain anti-Rh<sub>123</sub> derived from an interethnic plasma fraction prepared by other fraction of biological human manufacturers.

**Authors:** With an injection of native Rh<sub>123</sub> antibody (Rh<sub>123</sub>Ab) to the postpartum mother postnatally/abortus/mother, or in the recipient of transfusion accident, the patient antibody response to the foreign Rh<sub>123</sub> antibody cells, anti-Rh<sub>123</sub>.

Continuous reactions of  $\text{Rh}_2(\text{O})$  negative individuals gives  $\text{Rh}_2(\text{O})$  Lignum Globulosum (Phanes) are an important, of solid nature and mostly confined to the area of system. An occasional patient may react more strongly both locally and generally. A slight elevation of temperature has been noted in small numbers of positive reactions.

Fever, myalgia, lethargy, increased bilirubin levels and/or splenomegaly have been infrequently observed in individuals following multi-dose regimen after 10 consecutive intramuscular

**System** Windows 98 SE was used throughout due to reported systems of software problems in Windows.

Immune Serum (Globulin Otsuuki) has not been reported to produce hepatitis.

The QAM is to be given to the recipient in  
transformer accident or the participant teacher  
or postmaster's witness, only. It must not  
be given to the subject.

**Contraindications:** Bz<sub>2</sub>CO<sub>2</sub> Esters Chlorides (Fluorides) should not be administered to

1. An Rh(D) positive or D<sup>+</sup> positive individual
2. An Rh(D) negative patient who has inadvertently received an Rh(D) positive blood transfusion - the three months of delivery
3. A patient previously exposed to the Rh(D) blood issue

Supplies in package containing single-dose vial of EnoxAM and package containing 12 single-dose vials of EnoxAM.

**NOTE:** For complete prescribing information, see package insert.

# RhoGAM

## Recommendation for Usage of Units and Abbreviations

The Editorial Board of *Acta Haematologica* supports the recommendations of the International Committee for Standardization in Hematology for using the SI (Système International d'Unités) equivalents in haematological literature. The standard abbreviations used for measurements of weight, length, volume, and time (i.e.,

mg, cm, ml, min) are used without definition. The abbreviations listed below also require no preliminary definition. Please note that punctuation is not needed. When using other abbreviations, spell out the name in full at the first mention, and follow with the abbreviation in parentheses.

Entity	Abbreviation	Recommended SI equivalent	Not recommended
Red blood cell count	RBC	$10^9/l$	$10^9/mm^3$
White blood cell count	WBC	$10^9/l$	$10^9/mm^3$
Platelet count		$\times 10^9/l$	$\times 10^9/mm^3$
Reticulocyte count		% (or $\times 10^9/l$ )	% (or $\times 10^9/mm^3$ )
Haemoglobin	Hb	g/dl <sup>a</sup>	g/100 ml
Packed cell volume	PCV	ratio <sup>b</sup>	%
Mean cell volume	MCV	fL	
Mean cell haemoglobin	MCH	pg	
Mean cell haemoglobin concentration	MCHC	g/dl	%
Mean cell diameter	MCD	$\mu m$	
Sedimentation rate	ESR	mm in 1 h <sup>c</sup>	
Plasma haemoglobin	Plasma Hb	mg/l	mg/100 ml
Serum vitamin B		ng/l	
Serum folate		ng/l	
Serum iron		$\mu moles/l$	$\mu g/100 ml$
Total iron binding capacity	TIBC	$\mu moles/l$	$\mu g/100 ml$
Unsaturated iron binding capacity	UIBC	$\mu moles/l$	$\mu g/100 ml$
Transferrin		g/l	mg/100 ml
Serum haptoglobin		g/l	mg/100 ml
Plasma fibrinogen		g/l	mg/100 ml

<sup>a</sup> The concentration for Hb only is expressed as g/dl and not g/l or g/100 ml.

<sup>b</sup> No unit necessary. 1/l is implied.

<sup>c</sup> Indicate method only if other than Westergren.

# Treatment of Multiple Myeloma

Raymond Alexanian

University of Texas M.D. Anderson Hospital, Houston, Tex.

Multiple myeloma is a clonal malignancy of plasma cells that most often presents as a widespread tumor with bone destruction, anemia, recurrent infection, hypercalcemia and/or renal failure. Since the tumor is derived from a single clone, a specific immunoglobulin is produced which can be detected in about 96% of patients by simple electrophoretic techniques. Less than 10% of patients present with a localized plasmacytoma or in an indolent phase discovered because of an elevated serum protein or mild anemia in an asymptomatic person.

All patients with clinical complications secondary to myeloma or an unequivocally rising protein level require chemotherapy from the time of diagnosis. Before chemotherapy is initiated, optimal control of reversible medical complications, such as overt infection, is desirable. Treatment should not be delayed because of hypercalcemia, particularly since the therapeutic regimen will always include corticosteroids. For patients with bone pain, at least one course of chemotherapy is useful to reduce the generalized disease before local radiotherapy is considered, unless the latter is required for spinal cord compression or a long bone fracture.

The effectiveness of chemotherapy is best monitored with serial measurements of the serum myeloma protein concentration

and/or Bence Jones protein excretion. For IgA myeloma values less than 2,000 mg/dl measurements should be performed by direct immunoglobulin quantitation. It is easy to underestimate the true degree of tumor reduction if the changing catabolic rate for different IgG levels, the reduction of plasma volume with therapy and the effect of normal 'background'  $\gamma$ -globulin levels are not taken into account. Generally when such variables are evaluated, a reduction in an IgG spike from 4 to 2 g/dl (50% reduction) conforms more closely to a 75% reduction in tumor mass. Close attention should also be paid to the rate of reduction in the levels of myeloma protein. Since the halving time of serum myeloma protein production in patients responding to chemotherapy is 12 months or less, serial measurements which show a slower rate of decline during the first few months of treatment strongly suggest that a long-term remission will never develop.

## Remission Induction in Previously Untreated Patients

In 1969 the Southwest Oncology Group (USA) reported that an intermittent course of melphalan and prednisone given at 6-week intervals reduced myeloma tumor mass by more than 75% in approximately

45% of patients, resulting in a median survival for all patients of about 2 years. The addition of prednisone clearly improved the response rate by 20% and extended survival by about 5 months. These observations were soon confirmed by other centers and intermittent melphalan-prednisone therapy became a standard treatment for patients with multiple myeloma.

Over the last 10 years, a number of other drug combinations and regimens have been evaluated (table I). Investigators have looked at different alkylating agents, routes of administration, the efficacy of vincristine and adriamycin in the regimen and schedules of drug dose and treatment interval. At least three regimens combining an alkylating agent with vincristine, adriamycin and prednisone, administered at 3-week intervals, have improved the response rate to approximately 65% and the median survival time to 30 months [3] (table I). Regimens containing vincristine and adriamycin appeared to produce response rates 10–20% higher than combinations without these drugs. This may correlate with an increased growth fraction of plasma cells during the early months of treatment. Kinetic studies have suggested a higher fraction of proliferating cells in some patients during the first 6 months of treatment and during relapse.

Consequently the author considers intermittent courses of a vincristine (1 mg i.v.) – adriamycin (25 mg/m<sup>2</sup> IV) – cyclophosphamide (100 mg/m<sup>2</sup>/day for 4 days) – prednisone (60 mg/m<sup>2</sup>/day for 4 days) combination as the best regimen now available for previously untreated patients. Repeated courses should be continued at 3-week intervals in escalating doses to produce transient granulocytopenia between each course.

In both Southwest Oncology Group and Canadian drug trials, combinations of different alkylating agents gave no better results than those obtained with a single alkylating agent [3, 4]. In contrast, leukemia group B reported that combinations of alkylating agents prolonged survival in poor-risk patients but may have been harmful to those with fewer complications [6].

Recent improvements in survival for myeloma patients cannot be attributed fully to improved chemotherapy programs. Earlier diagnosis, a lower frequency of severe renal failure, superior control of infections and the ability to gain second remissions with drugs such as adriamycin may well influence survival statistics. Different definitions of risk status, responses to treatment, drug regimens, etc. may also lead to apparent differences in reported response rates. Important prognostic factors to consider in

Table I. Response and survival from different drug combinations in multiple myeloma

Treatment	Evaluable, n	Responsive, n	Response rate % evaluable	Median survival months
CAP	53	23	43	31
VCAP	83	54	65	31
VMCP	90	52	58	30
VBAP	47	30	64	28

See Alexanian *et al.* [3] for details of dose regimens and criteria for evaluating response. A = Adriamycin  
 B = BCNU C = cyclophosphamide M = melphalan P = prednisone V = vincristine.

the interpretation of any treatment include the size of the tumor mass before treatment and the presence of renal failure, hypocalcemia, or an IgA myeloma protein [1]. These factors clearly influence survival. With therapy the maximum degree of tumor reduction also has a major bearing on prognosis. Thus, the median survival in patients with a large tumor mass and no reduction in myeloma protein with therapy is about 1 year while in patients with a low tumor mass and disappearance of the abnormal protein it is about 4 years.

### Remission Maintenance

Until recently no information has been available as to the preferred approach to remission maintenance. Southwest Oncology Group studies have now shown that the median survival for responders given no treatment after 12 months of initial chemotherapy was similar to that for patients maintained indefinitely on melphalan-prednisone or BCNU prednisone [2]. This probably resulted from the high frequency (about 70%) of second remissions in patients relapsing while on no treatment. Even though the degree of second tumor reduction was usually less and the duration of remission shorter survival was the same. Therefore, patients in whom myeloma proteins disappear with therapy should not receive maintenance therapy inasmuch as they show a longer duration of remission (median 18 months). Patients with a persistent abnormal serum protein should continue on some form of regular maintenance therapy with a vincristine-alkylating agent-steroid combination. Other remission consolidation regimens incorporating azathioprine, BCG low-dose cytosine arabinoside, methotrexate, or

5-fluorouracil have shown no apparent benefit. Longer follow-up of the many patients now receiving levamisole is required before the value of this drug can be defined.

### Relapsing Patients

Unless death supervenes from a complication of chemotherapy or an unrelated disease process, all patients relapse eventually. A steadily rising level of abnormal protein of the same character as that present originally usually provides the best index of relapse. Median survival from the first return of the myeloma protein is about 9 months. Occasionally patients relapse with an increased Bence Jones protein excretion in relation to their tumor mass or an increase in bone lesions without a rise in serum protein. This suggests that some tumors may become more primitive during relapse with a reduced rate of globulin production per cell. Therefore, both the level of urinary Bence Jones protein and the size and number of lytic bone lesions should be evaluated regularly during remission.

Changes in plasma cell percentage have been less useful in evaluating disease progression. However recent studies suggest that cytophotometry studies of the bone marrow may improve the detection of relapse by demonstrating increased percentages of abnormal plasma cells with a high DNA and RNA content. When unexplained pancytopenia appears, the bone marrow should be carefully evaluated for the presence of a sideroblastic anemia or acute leukemia. These are late complications of alkylating agent therapy.

Little is known about the most effective treatment for relapsing patients. Studies by the Southwest Oncology Group have not

detected any consistent response to BCNU cyclophosphamide, hexamethylmelamine or several other new agents in patients resistant to melphalan prednisone given in maximum tolerated doses. When adriamycin was given alone or in combination with vincristine, prednisone and BCNU about 25% of relapsing patients achieved greater than a 50% reduction in tumor mass. This prolonged survival by about 9 months. In contrast, only 5% of patients unresponsive to previous chemotherapy improved. Further studies of more aggressive chemotherapeutic regimens are needed.

Human leukocyte interferon has recently shown therapeutic activity against multiple myeloma. In results reported from Sweden, 3 of 4 patients responded, while about 25% of patients of previously untreated or relapsing patients demonstrated 50% or greater reductions in tumor mass in studies at the M.D. Anderson Hospital [5]. As yet, little is known of the place and value of hemibody irradiation or total body irradiation with marrow transplantation in the induction and maintenance of long-term remissions.

### Unresponsive Patients

Provided pancytopenia is not severe, some form of standard chemotherapy should be continued indefinitely in most unresponsive patients in order to inhibit tumor growth. However when the calculated residual tumor mass is low enough that disease morbidity is unlikely about one-fourth of unresponsive patients can be followed without treatment until there is some evidence of disease progression. As with many patients achieving remission there is no obvious advantage from indefinite chemotherapy.

The therapeutic approach to patients with multiple myeloma has become more complex, more quantitative, and yet more rational. The clinician must consider whether any chemotherapy is indicated at all, the specific drug combination to be used, the duration that it should be continued, and the indications for adriamycin and other combinations. Serial electrophoretic studies must be used to quantitate changes in myeloma tumor mass. Only then can the different available strategies be applied most effectively for the individual patient.

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## Original Papers

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### Benign Bence Jones Gammopathy

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**Key Words.** Bence Jones proteinuria. Benign monoclonal gammopathy. Light chain disease.

**Abstract.** A case of isolated  $\lambda$  light chain proteinemia and proteinuria is reported. During a 42-month follow-up, no sign of myelomatosis or amyloidosis developed and Bence Jones proteinuria remained nearly of the same magnitude. This case of apparently idiopathic Bence Jones gammopathy, the first of  $\lambda$ -type so far reported, is added to similar observations to give further support to the contention that a benign counterparty to light-chain myeloma may exist.

#### Introduction

Bence Jones proteinuria is commonly regarded as a crucial point in the differential diagnosis between benign monoclonal gammopathy and myelomatosis. Significant elimination of monoclonal light chains in the urine is considered quite exceptional in benign gammopathy [13] as opposed to myeloma of which it is a well-known feature.

In myelomatosis, the imbalance in heavy and light-chain synthesis responsible of Bence Jones protein secretion is thought to be the expression of a process of plasma cell dedifferentiation [4, 5] and, therefore, by itself an index of malignancy. In some

10-20% of myelomas [11] this process is presumably more marked and the malignant cells are able to synthesize and to secrete Bence Jones proteins only. These light chain myelomas have a worse prognosis [5] showing a faster evolution and a higher frequency of amyloidosis, renal failure, osteolytic lesions and hypercalcemia. Nevertheless, 3 cases with isolated Bence Jones proteinuria, but no other evidence of myelomatosis have been recently recorded [3, 12].

In the present report we describe a similar case in which isolated monoclonal light chains were recognized in the urine and serum. Since no sign of malignant transformation or of amyloidosis has been discovered



over a 3½ year period, we consider this patient as a probable case of benign Bence Jones gammopathy

### Case Report

On July 7 1976, a 53-year-old woman was admitted to our hospital because of an attack of paroxysmal supraventricular tachycardia and angina pectoris. 40 years before, she had had a history of acute rheumatic fever and at the age of 35 an aortic valve disease had been found. On admission, the patient's dysrhythmia and angina promptly disappeared after administration of digitalis. Non-invasive cardiac examinations and cardiac catheterization confirmed the clinical diagnosis of aortic valve disease with prevalent aortic stenosis. Laboratory investigations showed hemoglobin 12.7 g/dl white blood cell count 7,300/mm<sup>3</sup> (differential normal) and erythrocyte sedimentation rate 4 mm/h. Serum electrolytes were in the normal range: sodium 144 mEq/l, potassium 3.8 mEq/l, chloride 96 mEq/l, calcium 9.6 mg/dl and phosphate 3.3 mg/dl. Cholesterol was 196 mg/dl and triglycerides 179 mg/dl. Liver function tests were normal. Total serum proteins were 6.40 g/dl with albumin 3.7 g/dl,  $\alpha_1$  globulin 0.3 g/dl,  $\alpha_2$ -globulin 0.7 g/dl,  $\beta$ -globulin 0.8 g/dl and  $\gamma$ -globulin 0.9 g/dl. Serum immunoglobulin quantitation showed the following values: IgG 628 mg/dl, IgA 124 mg/dl, IgM 48 mg/dl. No monoclonal spike was seen. BUN was 18 mg/dl and serum creatinine 1.08 mg/dl with a creatinine clearance of 92 ml/min. Re-

peated 24-hour urine specimens revealed proteinuria. It ranged from 1.50 to 2.50 g. In the urine sediment, numerous leukocytes and occasional hyaline casts were found. In the assumption of a concomitant pyelonephritic process, the patient underwent a fluid restriction study and an intravenous pyelography. Both gave normal results. A laboratory-required urine electrophoresis revealed a very weak fraction with the mobility of albumin and a sharp slow  $\gamma$ -globulin spike that immunoelectrophoresis identified as formed by monoclonal  $\lambda$  light chains (fig. 1). In the following days, total elimination of Bence Jones protein was estimated to range from 1.2 to 2.2 g/24 h. The same M protein was discovered in the serum by immunoelectrophoresis (fig. 1). No cryoglobulins were found. Complete X-ray skeletal survey and bone scans were negative. Bone marrow was normocellular except for slight plasmacytosis (6.8% of nucleated cells). Large polyploid plasma cells were seen. No chromosomal aberration was recognized. The patient was given digitalis and nitrates and was discharged from hospital. Close follow-up with bi-monthly clinical and laboratory evaluations was carried on (fig. 2). Elimination of free  $\lambda$ -light chains in the urine remained nearly of the same magnitude.

Slightly subnormal levels of IgG and IgM with IgA in normal range as well as monoclonal  $\lambda$ -light chains were constantly found in the serum. Various bone marrow aspirations showed normal or scantily increased concentrations of plasma cells. Bone marrow biopsy and rectal biopsy did not demonstrate amyloidosis. No sign of renal failure appeared. Bone scans and roentgenographic exam-

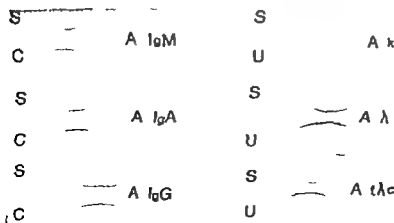


Fig. 1. Serum and urine immunoelectrophoresis. The serum (S) of the patient was compared with normal serum (C) for the three major immunoglobulin classes. Antisera against Bence Jones protein types  $\kappa$  and  $\lambda$  and against free  $\lambda$ -chains ( $\lambda$ -c.) disclosed monoclonal  $\lambda$  light chains in the serum (S) and urine (U) of the patient.

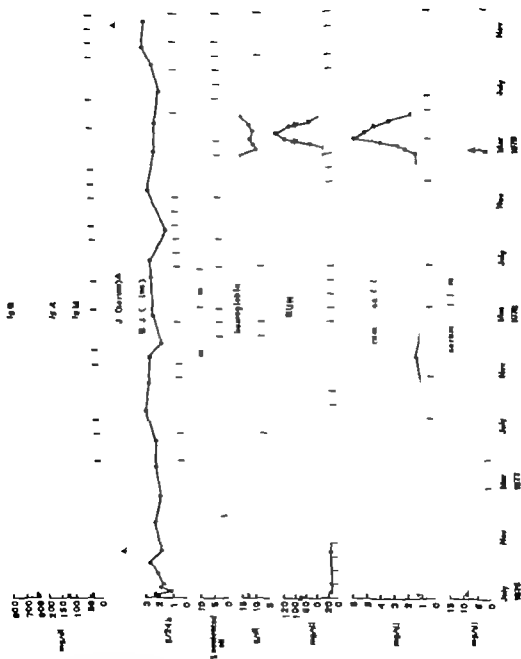


Fig. 2. Laboratory findings during the clinical follow-up of the patient. The date of the open heart operation is indicated (arrow).

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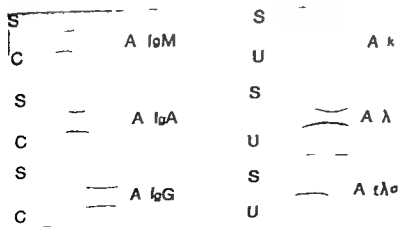


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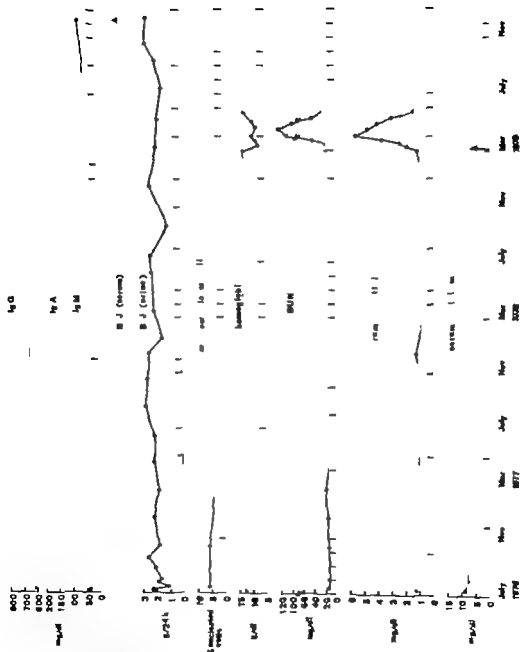


Fig. 1 Laboratory findings during the clinical follow-up of the patient. The date of the open heart operation is indicated (arrow).

Injections continued to be negative. In March 1979 the patient underwent the replacement of her aortic valve with a porcine heterograft. The operation was made more complicated by copious bleeding, acute myocardial infarction and two subsequent episodes of ventricular fibrillation, these latter being promptly overcome by electric shock. In the following days, atrial fibrillation with rapid ventricular rate, heart failure and a progressive impairment of renal function arose. BUN reached maximal values of 120 mg/dl and serum creatinine of 547 mg/dl. Hemoglobin ranged from 7.4 to 9.9 g/dl. The administration of blood and digitalis, together with a careful fluid balance, gradually resolved both cardiac and renal insufficiency. In May 1979 when the patient was discharged, BUN was decreased to 20 mg/dl and serum creatinine to 1.52 mg/dl. Hemoglobin was 12.2 g/dl. The histological examination of the aortic valve was negative for amyloid. In December 1979 the patient was checked for the last time. She continued to show the urine and serum protein abnormalities of light-chain disease but lacked other clinical evidence of myelomatosis.

## Methods

Urine and serum protein electrophoresis was performed on cellulose acetate membranes (barbital buffer pH 8.8) and stained with Ponceau S. The optical density of the bands was estimated by a Gelman ACD-18 densitometer. The amount of urinary proteins was evaluated by the sulfosalicylic acid method. Before electrophoretic analysis the urine specimens were concentrated, when required, up to 30 times by osmodialysis against polyethylene glycol 6000 (Merck, Schuchardt). Immunoelectrophoresis of serum and urine samples was performed on 2% agar plates (barbital buffer pH 8.2). Antisera against Ig, IgG, IgA, IgM, IgD, IgE, Bence Jones protein types K and  $\lambda$ , and free  $\lambda$  and  $\lambda$ -light chains were obtained from Behringwerke, Marburg/Lahn (FRG). The concentration of serum immunoglobulins was measured by single radial immunodiffusion. The total elimination of light chains was determined on the basis of 4-hour proteinuria and of the densitometry percentage of the urinary light-chain fraction.

## Discussion

Light-chain disease is the term commonly used to indicate a variant of multiple myeloma in which owing to a still more anaplastic process, malignant cells are able to produce Bence Jones proteins only. Our finding of the present case of apparently benign light-chain dyscrasia ( $\lambda$ -type) would seem to suggest that a benign counterpart to light-chain disease may exist. In 1974 a similar patient has been described [3]. This patient showed monoclonal light-chain proteinuria ( $\lambda$  type) nonassociated with serum M components and myelomatosis. Nevertheless, the idiopathic character of such a case of Bence Jones proteinuria is made rather questionable by the finding of antecedents of colonic carcinoma. The association of monoclonal gammopathies with this and other nonreticular neoplasias is, in fact, a well-documented phenomenon [1] although its biologic setting is not fully apparent. More recently 2 additional cases with isolated Bence Jones proteinuria (both of  $\lambda$  type) have been detected [12]. During a clinical follow up of only 2 and 7 months, respectively they did not show any evidence of myelomatosis.

Other exceptions to the axiom that the production of monoclonal light chains is virtually an index of malignancy are being recognized. The cases of 2 patients [6] who had had benign IgG monoclonal protein as well as significant Bence Jones proteinuria for more than 7 years and of another one [10] showing asymptomatic IgA and Bence Jones monoclonal gammopathy for a 6-year period have been recorded. Furthermore, in a follow-up study [7] immunoelectrophoresis demonstrated free light chains in the urine of 12 among 45 persons with benign monoclonal gammopathy.

From the above observations it is also inferred that Bence Jones proteins in at least some patients lack nephrotoxicity. In this regard it is of interest that our patient has excreted about 2 g of free light chains daily for more than 3 years without showing overt renal insufficiency. In addition, a water deprivation study, an intravenous pyelography and an open heart operation, the latter being followed by important hemodynamic imbalance, did not give rise to any irreversible impairment of her renal function.

Only prolonged periods of observation may indicate which cases of monoclonal gammopathy are likely to show malignancy. Waldenström [13] states that a globulin level stable for more than 1 year speaks in favor of benignity. For a final diagnosis of benign monoclonal gammopathy Hobbs [4] underlines the necessity for the overall clinical and laboratory picture to remain unchanged for at least 3 years. On the other hand, the development of classic myeloma has been recognized at intervals ranging from 15 to 24 years after the discovery of asymptomatic monoclonal hyperglobulinemia [8]. In our opinion, however, it seems very unlikely that in the case of light-chain disease, in particular of  $\lambda$ -type, the myelomatous proliferation does not manifest over a long period of time. Evidence has accumulated [2, 9] that myelomas producing only  $\lambda$ -light chains have by far a poorer prognosis than IgG, IgA or  $\kappa$ -myelomas. In a recent study 45 patients with  $\lambda$ -light chain disease, only 9 of whom untreated, showed a median survival from diagnosis of 10 months out of them 42% died within the first 6 months after diagnosis [11].

In the present case, in the absence of a specific treatment, isolated  $\lambda$ -light chain proteinemia and proteinuria have been present

for 42 months without any substantial change in their magnitude and without the development of myeloma or amyloidosis. We would like, therefore, to report our patient as a probable case of benign Bence Jones gammopathy to the best of our knowledge, the first  $\lambda$ -type gammopathy reported until now. Only large qualitative screenings of urinary protein abnormalities in otherwise healthy individuals will demonstrate whether benign Bence Jones gammopathy is merely a rare, though provocative entity or instead, a condition that occurs more frequently than expected.

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## Fc $\gamma$ and Fc $\mu$ Receptors in B Cell Neoplasms

### Correlation in the Developmental Stages

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**Key Words.** B-CLL, Fc $\gamma$  receptor, Fc $\mu$  receptor, Human B lymphocyte antigens, IgG-producing lymphoproliferative disorders, Macroglobulinemia, Plasma cell leukemia, Plasmacytoma, Surface Ig.

**Abstract.** Membrane receptors for the Fc portions of IgG (Fc $\gamma$  receptor) and IgM (Fc $\mu$  receptor) on leukemic cells in B cell neoplasms in various developmental stages were studied. In 6 out of 7 patients with B-CLL, leukemic cells had both receptors. In macroglobulinemia, leukemic cells had one or the other of Fc $\gamma$  and Fc $\mu$  receptors. In plasma cell leukemia and plasmacytoma, malignant cells had neither Fc $\gamma$  nor Fc $\mu$  receptors. The disappearance of Fc receptors appears to be associated with B cell differentiation.

### Introduction

The presence of receptors for the Fc portions of IgM (Fc $\mu$  receptors) on the surface of normal and leukemic T cells was described by Moretta *et al* [12, 13]. This new surface marker was also found on normal and leukemic B cells [7, 14]. Leukemic B cells also possess receptors for the Fc portions of IgG (Fc $\gamma$  receptor) [5]. It was postulated that the level of expression of the various B cell neoplasms can be correlated with different stages in B cell differentiation [16].

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In recent work, we examined Fc $\gamma$  and Fc $\mu$  receptors on neoplastic B cells derived from various developmental stages. Our findings strongly suggested that disappearance of Fc receptors is probably associated with B cell differentiation.

### Materials and Methods

#### *Patients*

We examined a total of 15 Japanese patients with B-CLL (7), IgG-producing lymphoproliferative disorders (1), macroglobulinemia (2), plasma cell leukemia (4), and plasmacytoma (1). All patients were referred to our laboratory. The diagnoses were based on clinical, laboratory and pathological criteria.



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Table II. Conventional cell surface markers

Case	Abnormal cell, %	EN RFC %	SIg %	EAC RFC %	HBLA %	EAIgG RFC (Fv7), %	EAIgM RFC (Fc2), %
1	96	1	2	6	87	28	86 (16)
2	98	1	3	5	83	78	18 (38)
3	94	8	2	not tested	not tested	64	83 (54)
4	98	6	78 (M, D, r)	9	not tested	40	31 (17)
5	95	5	92 (M, )	not tested	87	92	58 (37)
6	86	12	84 (O )	86	not tested	76	26 (82)
7	90	9	69 (O )	not tested	85	46	0 (2)
8	83	5	24 (O )	46	not tested	48	12 (59)
9	97	3	70 (M, D, r)	6	not tested	3	35 (23)
10	82	8	26 (M, r)	not tested	not tested	80	2 (3)
11	82	16	8	4	not tested	14	5 (not tested)
12	83 (TD) <sup>1</sup>	5	8	not tested	not tested	5	7 (1)
13	80 (TD)	5	10	not tested	12	5	1 (12)
14	83 (TD)	1	9	not tested	10	7	0 (2)
15	75 (tumor)	2	3	3	not tested		0 (not tested)
Normal (15)		73 ± 8	13 ± 5	26 ± 5	10 ± 3	30 ± 8	(52 ± 19)

T-cell-depleted cell population.

Letters in parentheses indicate monoclonality.

Numbers in parentheses indicate the score after overnight culture.

reagent reagents used were the same as described above.

Human B lymphocyte antigens were detected by rabbit anti-B cell serum (Alpha Gamma Labs, Sierra Madre, Calif. lot. 09236) [3]. An indirect immunofluorescent test was performed as described by *Bulheng et al.* [2].

## Results

Summaries of patients profiles are shown in table I. In case 8 (previously reported by *Akasaka et al.* [1]), the serum contained 5 g/dl monoclonal IgG, however clinical pictures resembled those of macroglobulinemia as in the cases reported by *Respetti et al.* [15] and *Towse et al.* [17].

The results of conventional cell surface

markers are shown in table II. In a case with a low leukocyte count, T-cell-depleted cell populations were examined. More than 75% of the cells obtained were abnormal cells (table II).

Leukemic cells from 4 out of 7 patients with B-CLL bore monoclonal SIg. In other patients with B-CLL, however they did not express SIg. In this paper we considered these cases as B-CLL because clinical pictures and morphologic studies of leukemic cells differed little from those of typical cases with B-CLL and leukemic cells possessed human B lymphocyte antigen. These exceptional cases have been reported by several authors [9-11].

In 6 patients with B-CLL (cases 1-6) and in 1 patient with IgG-producing lym-

### Culture of Cell Suspensions

Normal peripheral blood lymphocytes and leukemic cells were separated by Ficoll sodium metrizoate gradient centrifugation (400 g for 30 min) [18]. Interface cells were washed twice with phosphate-buffered saline (0.15 M pH 7.6), once with TC 199 medium (Nissui, Tokyo) and suspended in TC 199 containing 20% fetal calf serum (Gibco lot, C175416). Surgically removed tumors were cut into small pieces with scissors in TC 199 medium. Single cell suspensions were obtained by removing large aggregates (500 rpm for 2 min).

T cells and T-depleted cells were separated by neuraminidase treated sheep red blood cell (En) rosettes through a Ficoll-sodium metrizoate gradient [19]. One million lymphocytes were cultured in a disposable round-bottomed tube (Falcon 2006) in 1 ml TC 199 supplemented with 20% fetal calf serum in a humid atmosphere of 5% CO<sub>2</sub> at 37 °C. In all cases, viable cells exceeded 95% as determined by the trypan blue dye exclusion test.

### Cell Surface Markers

For demonstration of Fcγ and Fcγ<sub>2</sub> receptors, we used the methods described previously [19] with a slight modification. Briefly IgM was purified from a rabbit anti-ox red blood cells (ORBC) antiserum containing mainly IgM antibody [10] by Sephadex G 200 gel filtration. IgG was purified by DE 52 cellulose chromatography from an antiserum raised in a rabbit by multiple intravenous injection of intact ORBC. Each antiserum gave a single precipitin line in immunoelectrophoresis against the whole serum. ORBC were sensitized with subagglutinating titer of anti-ORBC IgM or IgG for 30 min at 37 °C (EAIgM, EAIgG). Cells were washed three times and resuspended in TC 199 medium supplemented with 20% fetal calf serum to give a 1% suspension. 150 μl of each indicator cells were mixed with equal volume of leukemic cells (2–3 × 10<sup>4</sup>/ml) centrifuged for 5 min at 200 g and kept at 4 °C for 1 h. The pellet was very gently suspended and at least 200 cells were counted in a hemocytometer.

Spontaneous En rosette formation of T cells was performed as described previously [19].

Complement receptors were detected by use of ORBC coated with a rabbit anti-ORBC IgM antiserum and a human complement (EAC) by a slight modification of the method previously described [18].

Surface-bound immunoglobulins (SIg) were detected by a direct immunofluorescent method as described elsewhere [18]. A fluorescein-conjugated rabbit antiserum to human immunoglobulins and antiserums monospecific for γ, μ, α, κ, λ and I determinants (Behringwerke, Marburg/Lahn) were used. Each antiserum was absorbed with human red blood cells, optimally diluted with phosphate-buffered saline and filtered through a Millipore membrane (0.45 μm). Cells were examined after an overnight culture to remove cytophilic immunoglobulins. Cells with membrane fluorescence were enumerated using a Zeiss ultraviolet microscope with a vertical illuminator. Monoclonality of SIg is defined when the over 80% of immunofluorescent positive cells (by antihuman immunoglobulins) reacted with a single reagent.

Intracytoplasmic immunoglobulins were detected as described elsewhere [19]. Immunofluorescence

Table I. Clinical diagnoses and profiles of 15 patients

Case	Age years	Sex	Diagnosis	WBC 10 <sup>9</sup> /l
1	52	M	B-CLL	27.5
2	72	M	B-CLL	43.9
3	61	M	B-CLL	22.5
4	78	M	B-CLL	77.5
5	79	M	B-CLL	33.8
6	72	M	B-CLL	2.2
7	53	M	B-CLL	24.5
8	79	M	IgG-CLL	7.7
9	64	F	macroglobulinemia	42.5
10	58	F	macroglobulinemia	6.9
11	45	M	PC leukemia (nonsecretory)	22.1
12	54	F	PC leukemia (IgG λ)	11.9
13	35	M	PC leukemia (IgG κ)	13.2
14	62	F	PC leukemia (IgA, κ)	15.1
15	69	M	plasmacytoma (IgA, κ)	4.5

IgG-producing lymphoproliferative disorders.  
Plasma cell leukemia.

Table II. Conventional cell surface markers

Case	Abnormal cell, %	EN RFC %	Sig %	EAC RFC %	HLA %	EAIgG RFC (Fv7), %	EAIgM RFC (Fv9), %
1	96	1	2	6	87	28	86 (16)
2	96	1	3	5	83	78	18 (38)
3	94	8	2	not tested	not tested	64	80 (34)
4	98	6	78 (M, D, x)	9	not tested	40	31 (17)
5	95	5	92 (M, )	not tested	87	92	58 (37)
6	86	12	84 (G, )	86	not tested	79	26 (82)
7	90	9	89 (G, x)	not tested	85	46	0 (2)
8	85	5	24 (G, x)	46	not tested	48	12 (39)
9	97	3	70 (M, D, x)	6	not tested	3	35 (23)
10	82	8	26 (M, x)	not tested	not tested	80	2 (3)
11	82	16	8	4	not tested	14	5 (not tested)
12	85 (TD) <sup>1</sup>	5	8	not tested	not tested	5	7 (1)
13	80 (TD)	5	10	not tested	12	5	1 (12)
14	85 (TD)	1	9	not tested	10	7	0 (2)
15	75 (tumor)	2	3	3	not tested	2	0 (not tested)
Normal (15)		73 ± 8	13 ± 5	26 ± 5	10 ± 3	30 ± 8	(52 ± 19)

T-cell-depleted cell population.

Letters in parentheses indicate monoclonality.

Numbers in parentheses indicate the score after overnight culture.

reagent reagents used were the same as described above.

Human B lymphocyte antigens were detected by rabbit anti-B cell serum (Alpina Gamma Lab., Sierra Madre, Calif. lot 09236) [3]. An indirect immunofluorescent test was performed as described by Buhring *et al.* [3].

## Results

Summaries of patients profiles are shown in table I. In case 8 (previously reported by Akasaka *et al.* [1]) the serum contained 5 g/dl monoclonal IgG; however clinical pictures resembled those of macroglobulinemia as in the cases reported by Resegotti *et al.* [15] and Turz *et al.* [17].

The results of conventional cell surface

markers are shown in table II. In a case with a low leukocyte count, T-cell-depleted cell populations were examined. More than 75% of the cells obtained were abnormal cells (table II).

Leukemic cells from 4 out of 7 patients with B-CLL bore monoclonal Sig. In other patients with B-CLL, however they did not express Sig. In this paper, we considered these cases as B-CLL because clinical pictures and morphologic studies of leukemic cells differed little from those of typical cases with B-CLL and leukemic cells possessed human B lymphocyte antigen. These exceptional cases have been reported by several authors [9-11].

In 6 patients with B-CLL (cases 1-6) and in 1 patient with IgG-producing lym-

### Culture of Cell Suspensions

Normal peripheral blood lymphocytes and leukemic cells were separated by Ficoll-sodium metrizoate gradient centrifugation (400 g for 30 min) [18]. Interface cells were washed twice with phosphate-buffered saline (0.15 M pH 7.6), once with TC 199 medium (Nissui, Tokyo) and suspended in TC 199 containing 20% fetal calf serum (Gibco lot C175416). Surgically removed tumors were cut into small pieces with scissors in TC 199 medium. Single cell suspensions were obtained by removing large aggregates (500 rpm for 2 min).

T cells and T-depleted cells were separated by neuraminidase treated sheep red blood cell (En) rosettes through a Ficoll sodium metrizoate gradient [19]. One million lymphocytes were cultured in a disposable round-bottomed tube (Falcon 2006) in 1 ml TC 199 supplemented with 20% fetal calf serum in a humid atmosphere of 5% CO<sub>2</sub> at 37 °C. In all cases, viable cells exceeded 95% as determined by the trypan blue dye exclusion test.

### Cell Surface Markers

For demonstration of Fcγ and Fcε receptors, we used the methods described previously [19] with a slight modification. Briefly IgM was purified from a rabbit anti-ox red blood cells (ORBC) antiserum containing mainly IgM antibody [10] by Sephadex G 200 gel filtration. IgG was purified by DE 52 cellulose chromatography from an antiserum raised in a rabbit by multiple intravenous injection of intact ORBC. Each antiserum gave a single precipitin line in immunoelectrophoresis against the whole serum. ORBC were sensitized with subagglutinating titer of anti-ORBC IgM or IgG for 30 min at 37 °C (EAIgM, EAIgG). Cells were washed three times and resuspended in TC 199 medium supplemented with 20% fetal calf serum to give a 1% suspension. 150 μl of each indicator cells were mixed with equal volume of leukemic cells (2–3 × 10<sup>6</sup>/ml), centrifuged for 5 min at 200 g and kept at 4 °C for 1 h. The pellet was very gently suspended and at least 200 cells were counted in a hemocytometer.

Spontaneous En rosette formation of T cells was performed as described previously [19].

Complement receptors were detected by use of ORBC coated with a rabbit anti-ORBC IgM antiserum and a human complement (EAC) by a slight modification of the method previously described [18].

Surface-bound immunoglobulins (SIg) were detected by a direct immunofluorescent method as described elsewhere [18]. A fluorescein-conjugated rabbit antiserum to human immunoglobulins and antisera monospecific for γ, μ, α, δ, κ and λ determinants (Behringwerke, Marburg/Lahn) were used. Each antiserum was absorbed with human red blood cells, optimally diluted with phosphate-buffered saline and filtered through a Millipore membrane (0.45 μm). Cells were examined after an overnight culture to remove cytoplasmic immunoglobulins. Cells with membrane fluorescence were enumerated using a Zeiss ultraviolet microscope with a vertical illuminator. Monoclonality of SIg is defined when the over 80% of immunofluorescent positive cells (by antihuman immunoglobulins) reacted with a single reagent.

Intracytoplasmic immunoglobulins were detected as described elsewhere [19]. Immunofluo-

Table I. Clinical diagnoses and profiles of 15 patients

Case	Age years	Sex	Diagnosis	WBC × 10 <sup>9</sup>
1	52	M	B-CLL	27.5
2	72	M	B-CLL	43.9
3	62	M	B-CLL	22.5
4	78	M	B-CLL	27.5
5	79	M	B-CLL	33.8
6	72	M	B-CLL	22.2
7	53	M	B-CLL	4.5
8	79	M	IgG-CLL	7.7
9	64	F	macroglobulinemia	42.5
10	58	F	macroglobulinemia	6.9
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1 IgG-producing lymphoproliferative disorders.

2 Plasma cell leukemia.

Table II. Conventional cell surface markers

Case	Abnormal cell, %	EN RFC %	SIg %	EAC RFC %	HLA %	EA1gO RFC (Pv), %	EA1gM RFC (Fca), %
1	96	1	2	6	87	28	86 (16) <sup>a</sup>
2	98	1	3	5	83	78	18 (38)
3	94	8	2	not tested	not tested	64	63 (54)
4	98	6	78 (M, D, s)	9	not tested	40	31 (17)
5	95	5	92 (M )	not tested	87	92	58 (37)
6	86	12	84 (G, s)	86	not tested	76	26 (82)
7	90	9	69 (G )	not tested	85	46	0 (2)
8	85	9	24 (G s)	46	not tested	48	12 (99)
9	97	3	70 (M, D )	6	not tested	3	35 (23)
10	82	8	26 (M, s)	not tested	not tested	80	2 (7)
11	82	16	8	4	not tested	14	5 (not tested)
12	85 (TD)	5	8	not tested	not tested	5	7 (1)
13	80 (TD)	5	10	not tested	12	5	1 (12)
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Human B lymphocyte antigens were detected by rabbit anti-B cell serum (Alpha Gamma Labs, Sierra Madre, Calif. lot. 09236) [5]. An indirect immunofluorescent test was performed as described by *Bilhou et al* [3].

## Results

Summaries of patients profiles are shown in table I. In case 8 (previously reported by *Akasaka et al* [1], the serum contained 5 g/dl monoclonal IgG, however clinical pictures resembled those of macroglobulinemia as in the cases reported by *Resegotti et al* [15] and *Turcz et al* [17].

The results of conventional cell surface

markers are shown in table II. In a case with a low leukocyte count, T-cell-depleted cell populations were examined. More than 75% of the cells obtained were abnormal cells (table II).

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In 6 patients with B-CLL (cases 1-6) and in 1 patient with IgG-producing lym-

phoproliferative disorders (case 8) leukemic cells formed rosettes with EAiG and EAiGM. In 1 patient with B-CLL (case 7) and in 1 patient with macroglobulinemia (case 10) leukemic cells formed rosettes with only EAiG. In the other case of macroglobulinemia (case 9) these cells formed rosettes only with EAiGM.

In contrast to the above findings pathological cells from patients with plasma cell leukemia and plasmacytoma did not form rosettes neither with EAiG nor with EAiGM.

### Discussion

We found evidence that leukemic cells from patients with B-CLL, IgG-producing lymphoproliferative disorders and macroglobulinemia formed rosettes with either EAiG or EAiGM and sometimes with both. These cells were not apparently plasma cells, because less than 4% of them contained intracytoplasmic immunoglobulins as defined by fluorescein-conjugated anti human immunoglobulins (data not shown).

Characterization of Fc $\mu$  receptor-bearing II cells have been discussed by several authors [4-8]. Our data showed that Fc $\mu$  receptors were present on SIg (-) and SIg (+) II cells. Furthermore Fc $\mu$  receptors were present on leukemic cells which might involve a differentiation into plasma cells. Thus Fc $\mu$  receptors are probably present on various B cell subpopulations.

The reports regarding the Fc $\mu$  receptors on plasma cells have not ever published. The Fc $\gamma$  receptors on plasma cells were controversial in mice. In humans, Fc $\gamma$  receptors were not detected on a plasma cell leukemia line [6]. However Wood and Amare [20] detected Fc $\gamma$  receptors on plas-

ma cells in bone marrow aspirates, using highly sensitized sheep red blood cells. We detected neither Fc $\gamma$  nor Fc $\mu$  receptors on neoplastic plasma cells. Although we have no adequate explanation for these discrepancies, one possibility is that plasma cells in the leukemic phase are transformed to a greater extent than are those in bone marrow aspirates.

We found that Fc $\gamma$  and Fc $\mu$  receptors disappear along with B cell differentiation. Hitherto, functions of Fc receptors were unknown. B lymphocytes are activated by the Fc region of IgG [2]. Thus it is likely that Fc receptors are required for B lymphocytes to mature into antibody-producing cells and unnecessary for plasma cells which have already matured.

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## Effect of Daily Administration of Cytotoxic Drugs on the Erythroid and Granulocytic Repopulating Ability of Rat Bone Marrow

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**Key Words.** Ability repopulating Administration daily Busulphan  
Cyclophosphamide Dimethylmyleran

**Abstract.** The erythroid and granulocytic repopulating ability of rat bone marrow has been measured after daily administration of three alkylating agents: dimethylmyleran, busulphan and cyclophosphamide. Three different patterns of response were observed. The results emphasize the variability of the response of haemopoietic tissue to repeated administration of different cytotoxic drugs and provide further evidence that erythroid and granulocytic repopulating assays measure different populations of precursor cells.

### Introduction

The effect of single doses of cytotoxic drugs on haemopoietic progenitor cells has been the subject of numerous investigations. However, despite the fact that multiple-dose regimes are commonly employed in the clinic, little is known about the response of haemopoietic tissue during repeated administration of these drugs. In contrast, the effect of daily or continuous doses of  $\gamma$ -irradiation has been well documented. The survival curves of stem cell populations measured by the spleen colony assay [Wu and Lajtha 1975], the erythroid repopulating assay and the erythropoietin response [Blackett, 1967; Porteous and Lajtha 1966; Blackett and Adams 1972] generally exhibit a plateau

which suggests that the increased cell loss from these compartments is compensated, at least to some extent, by increased cellular proliferation.

The purpose of the present study was, therefore, to compare the changes in the erythroid and granulocytic repopulating ability of rat bone marrow during daily treatment with three cytotoxic drugs, namely dimethylmyleran, busulphan and cyclophosphamide.

### Materials and Methods

Groups of 5 male F1 hybrid rats (10-13 weeks old) of the August and Marshall strains were injected daily with either dimethylmyleran (0.5 mg/kg), busulphan (1 mg/kg) or cyclophos-

phamide (20 mg/kg). Dimethylmyleran was dissolved in propylene glycol and diluted with saline immediately prior to injection. Busulphan was dissolved in dimethyl sulphoxide and then diluted 1 part in 9 parts acetone oil. Cyclophosphamide was dissolved in saline. All injections were by the intraperitoneal route.

The measurement of erythroid and granulocytic repopulating ability in rats has been previously described by Connable and Blackett (1972). The granulocytic repopulating ability was measured by injecting bone marrow from the treated rats intravenously into groups of 7 recipients that had received doses of 4.75 mg/kg dimethylmyleran 24 h previously. The number of nucleated cells injected was equivalent to between 0.2 and 2.0 femurs per recipient. 13 days after transplantation each recipient was injected with 60  $\mu$ g *Salmonella typhosa* endotoxin and, 8 h later, total nucleated blood count and differential were performed. Endotoxin produces a neutrophilia by mobilising the marrow mature neutrophil pool and the size of this compartment provides an index of granulocytic repopulation. To allow comparison between different groups, the mean neutrophil response for each group was normalised to 'per femur injected'.

The erythroid repopulating ability was measured by injecting bone marrow cells (equivalent to between 0.1 and 1.0 femur) intravenously into recipients that had received 5.8 mg/kg dimethylmyleran 24 h previously. 7 days after transplantation 1  $\mu$ Ci  $^{59}\text{FeCl}_3$  in 1% sodium citrate was injected intraperitoneally and blood sample was collected 28 h later. The percentage of the injected dose incorporated into circulating red blood cells provides measurement of erythroid repopulation. Again, comparison between groups was achieved by normalising to 'radioactive iron uptake per femur injected'.

The granulocytic and erythroid repopulating assays were usually performed in parallel using marrow from the same groups of donor rats. In each experiment, in addition to the groups receiving cells from treated donors, one group of 7 recipients received normal bone marrow cells and another group was not injected with cells. These two groups of recipients enabled the repopulating ability of bone marrow from the treated animals to be expressed as fraction of that for normal bone marrow. The recovery of endogenous erythropoietic and granulopoietic in the animals

treated with dimethylmyleran but no bone marrow was less than 10% of the controls which received marrow cells. Results were expressed as repopulating ability as fraction of control  $\pm$  standard error.

## Results

Following daily doses of dimethylmyleran, erythroid and granulocytic repopulating ability decreased exponentially at similar rates and reached a level of about 1% of normal on day 12 (fig. 1a). In contrast, daily administration of busulphan had a considerably greater effect on granulocytic repopulating ability compared to erythroid repopulating ability (fig. 1b). Granulocytic repopulating ability decreased to a value of about 2% of normal after 6 daily injections and was undetectable at later times. The erythroid repopulating ability was 24% on day 6 and about 2% of normal after 12 daily doses of busulphan.

Daily injection of cyclophosphamide decreased the erythroid repopulating ability to less than 20% of normal by day 5 after which there was an increase to 50% of normal on day 12 (fig. 1c). The granulocytic repopulating ability decreased less rapidly and reached a plateau of about 25% of normal at 10 days.

## Discussion

The results presented in this report clearly demonstrate the variability of the responses of progenitor cells in haemopoietic tissue following daily administration of different cytotoxic drugs. In the case of cyclophosphamide, an initial reduction in repopulating ability was followed by a pro-

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### Materials and Methods

Groups of 5 male F1 hybrid rats (10-13 weeks old) of the August and Marshall strains were injected daily with either dimethylmyleran (0.5 mg/kg), busulphan (1 mg/kg) or cyclophosph-

[Gregory *et al.* 1971] or melphalan [Millar *et al.*, 1978]. This action, which is most probably mediated by an effect on the haemopoietic microenvironment is presumably not shared by dimethylmyleran and busulphan, and therefore recovery after these agents is delayed.

It has previously been shown that erythroid repopulating cells proliferate more rapidly than the corresponding granulocytic population [Constable and Blackett 1972]. The greater effect of daily busulphan on granulocytic repopulating ability compared to erythroid repopulating ability is therefore consistent with the view that this agent has a preferential effect on slowly dividing cells [Dunn, 1972]. This observation may have clinical implications since a selective action on slowly proliferating granulocytic precursor cells could account for the preferential effect of busulphan on granulocytic cells in patients with chronic myeloid leukaemia. The failure to detect a difference between the effect of daily doses of dimethylmyleran on erythroid and granulocytic repopulating ability was not unexpected because the action of this drug is known to be independent of target cell proliferation rate [Dunn, 1972].

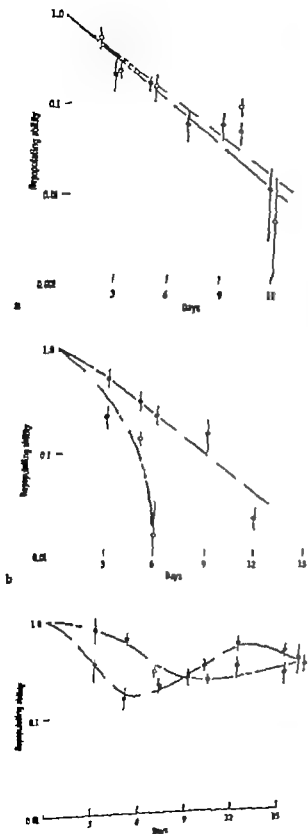
Measurement of repopulating ability provides a functional index of progenitor cell survival after cytotoxic drugs. Differences in the sensitivity of erythroid and granulocytic repopulating cells and pluripotent stem cells (CFUs) after single doses of cytotoxic agents indicate that these are distinct populations of precursor cells and it has been suggested that the repopulating cell compartments are composed of committed stem cells [Dunn and Constable, 1973]. Further evidence for the separate identity of erythroid and granulocytic repopulating cells has been provided by the present study

Since it is not clear whether committed repopulating cells are capable of extensive self renewal, the precise role played by these populations in promoting the long-term repopulation of bone marrow following drug treatment is not well defined. However, irrespective of their self-renewal capacity repopulating cells provide an output of mature cells at times which precede the permanent repopulation of haemopoietic tissue by the CFUs. This recovery could be transient but nevertheless critical for survival in the period prior to the full resumption of mature cell production.

The results that have been presented demonstrate some of the factors that influence the response of haemopoietic progenitor cells to repeated doses of cytotoxic drugs. They show that different drugs produce widely different effects and that this reflects not only the amount of cell kill but also the ability of the drug to influence the haemopoietic microenvironment and the feedback control mechanisms which regulate the proliferation of progenitor cells.

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nounced recovery phase despite continued daily exposure to the drug. This pattern is similar to that which is known to develop during treatment with continuous irradiation [Blackett 1967] and is probably due to the development of new steady-state kinetics whereby the increased cell loss is balanced by increased proliferation and cell production. However no such response was noted in the case of dimethylmyleran and busulphan. Both of these agents produced steady decreases in erythroid and granulocytic repopulating ability and there was no evidence of a plateau above the 0.01 level of survival.

The reason for this difference is not clear. In the case of irradiation, it is known that the level at which the survival of stem cells reaches a plateau depends on the daily dose rate [Yu and Lajtha 1975]. In general, the higher the dose rate, the greater the rate of cell loss and the lower the level at which compensatory changes occur. However the doses of the three drugs used in this study produced similar effects on erythroid repopulating ability following 5 daily injections, which suggests that the different patterns of response during repeated administration of these agents was not due to differences in the rate of cell loss from the progenitor cell compartments. A more likely explanation is that the response after repeated cyclophosphamide administration is related to the known capacity of this agent to accelerate haemopoietic recovery in animals treated with other cytotoxic agents, e.g. irradiation.

Fig. 1. Effect of daily administration of dimethylmyleran (0.5 mg/kg/day: a), busulphan (1 mg/kg/day: b) and cyclophosphamide (20 mg/kg/day: c) on granulocytic repopulating ability (O) and erythroid repopulating ability (●). Vertical bars represent standard error.

## Erythropoietic Changes Effected by Foreign Serum in the Mouse

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**Key Words.** Erythropoietic microenvironment Foreign serum Mouse erythropoiesis

**Abstract.** Injection of foreign serum into mice increases erythrocyte formation as evaluated by ferrokinetics studies. When erythropoiesis is depressed either by fasting or plethora, the wave of erythropoiesis that follows a transient increase of endogenous erythropoietin is clearly enhanced by pretreatment of the recipients with foreign serum. The response includes a restoration of the responsiveness of the spleen of fasted mice to endogenous erythropoietin stimulation. These changes seem related to an effect of foreign serum on the transition of primitive hematopoietic progenitors into erythroid-committed progenitors.

In the steady state erythropoiesis is presumably regulated by endogenously formed erythropoietin (EPO). However the role of the hormone as regulator of the process has become controversial as a number of clinical and experimental observations do not appear to fit this unified theory [6, 8, 9].

Injection of foreign serum (FS) into mice produces an increment of splenic erythropoiesis [1]. This effect has been ascribed to an increase in the pool of primitive hematopoietic progenitors (HSC) suggesting that some stages of erythropoiesis may be influenced by non-EPO mechanisms. The purpose of this study therefore, was to investi-

gate further the erythropoietic changes effected by injection of FS in the mouse.

### Material and Methods

F C58/FWD female mice 8-10 weeks old were used for all studies. FS was sheep serum separated at 4°C from freshly obtained blood. Donors were 4 young adult sheep with hematocrits in the range of 43-46. In order to eliminate possible hemolytic effect of FS, each serum was absorbed with an equal volume of packed mouse erythrocytes. Subsequently all samples were inactivated at 56°C for 30 min, centrifuged, pooled and stored frozen until use. In previous experiments the sera were found equally active when tested separately.

The study was carried out in normal, fasted and transfused plethoric mice. Fasting was started

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## Results

The drop in hematocrit in normal mice following intravenous injection of 0.5 ml of absorbed FS was significantly smaller (1.3%) when compared to the drop caused by injection of nonabsorbed serum (2.9%). Table I shows the variation of the hematocrits at various intervals after the second injection of absorbed serum.

In normal mice the coefficient of correlation between the values of hematocrits and percents of RBC  $^{59}\text{Fe}$  uptake determined at 72 h after the second injection of FS showed no significant correlation ( $r = 0.23$ ). In plethoric mice the polycythemic status remained unchanged throughout the experimental period.

Table II shows the effects of FS on spleen weight and on the  $^{59}\text{Fe}$  uptake by spleen, both femurs and circulating erythrocytes. Injection of FS promoted erythropoiesis as estimated by the increase in radioiron incorporation into circulating erythrocytes and into spleen, albeit the fraction of  $^{59}\text{Fe}$  taken up by the bone marrow showed a consistent fall. Changes in the spleen weight closely paralleled the variations in  $^{59}\text{Fe}$  utilization by the organ.

The effect of FS on the erythropoiesis of fasted and polycythemic mice is shown in figure 1. Fasting and induced plethoria resulted in a similar reduction of  $^{59}\text{Fe}$  uptake. In both cases radioiron utilization was almost completely abolished in the splenic area while the bone marrow retained about 25% of its normal activity (panel A). A different pattern of response was seen with a brief increase of endogenous EPO induced by injection of CO. In fasted mice the increase in  $^{59}\text{Fe}$  utilization was restricted to the medullary area while the spleen fraction show-



Fig. 1. Effects of FS on the erythropoiesis of fasted and polycythemic mice. Bars in the panels represent  $^{59}\text{Fe}$  uptake by the spleen (open bars), both femurs (striped bars) and circulating red blood cells (closed bars). A = Depressed untreated controls; B = response to erythropoietin alone; C = treatment with FS alone, and D = stimulation with erythropoietin after treatment with FS. Heights of bars represent values in each experimental condition and are expressed as percent of values in normal controls injected with isotonic saline solution which are taken as 100%.

ed a negligible change. This pattern of response sharply contrasts with that found in polycythemic mice where increments occurred in both areas (panel B).

Treatment of polycythemic and fasted mice with FS (panel C) failed to produce a statistically significant increase of  $^{59}\text{Fe}$  uptake in either the splenic or medullary fractions. However, when FS-treated mice were subsequently submitted to endogenous EPO stimulation, the splenic lack of sensitivity to



on day 1 and maintained throughout the experimental period. Mice were kept in individual cages and tap water was available *ad libitum*. Polycythemia was induced by intraperitoneal injection of 1 ml of isologous washed erythrocytes on day -2.

Groups of normal, fasted and polycythemic recipients received two 0.5 ml subcutaneous injections of FS on days 1 and 2. On day 3 groups of polycythemic and fasted animals were injected with 0.36 cm<sup>3</sup>/g body weight of carbon monoxide in order to stimulate endogenous formation of EPO [5]. On day 5 half a  $\mu$ Cl of <sup>55</sup>Fe was given intravenously and 3 h later percent of the injected radioiron taken up by spleen, both femurs and circulating erythrocytes was measured. <sup>55</sup>Fe uptake by circulating RBC was calculated with an assumed blood volume of 7% of body weight in plethoric mice and 6% in normal and fasted animals. Groups serving as controls received 0.85% NaCl in an equivalent volume at times when experimental groups were injected. Hematocrits were measured at 12, 24 and 72 h after the second injection of FS.

In other experiments, FS was preincubated at 37 °C for 30 min with anti-EPO serum, sufficient to neutralize the erythropoietic activity of 0.05 U/ml of EPO. Effectiveness of the antiserum to remove erythropoietin activity was tested against a sample of FS containing 0.1 U/ml of EPO. Recipients in these experiments were normal mice and

Table I. Effect of FS on hematocrit (mean  $\pm$  SD)

Time after FS injection h	Number of mice	Hematocrit		
		normal mice	polycythemic mice	fasted mice
12	9	48.2 $\pm$ 1.18	65.5 $\pm$ 2.0	48.7 $\pm$ 2.1
24	10	48.9 $\pm$ 1.60	64.2 $\pm$ 2.0	51.4 $\pm$ 1.3
72	10	47.8 $\pm$ 1.9	63.8 $\pm$ 1.1	54.2 $\pm$ 1.8
Controls		49.2 $\pm$ 1.5	64.2 $\pm$ 1.4	51.2 $\pm$ 1.2

Control mice were injected with isotonic saline solution.

controls were performed in groups injected with saline solution. Other controls were mice injected with saline solution containing the same amount of anti-EPO serum (w/v) used to neutralize FS.

The effects of FS on the number of HSC was assessed by measuring the number of colony-forming units (CFU) per 10<sup>6</sup> spleen cells by the transplant method as previously described [7]. Prospective cell donors were either normal or fasted mice. Deprivation of food in the latter was started on day 1 with free access to water. AB donors received 0.5 ml of FS on days 1 and 2. Spleen cells for transplant were harvested on day 5.

Table II. Effect of various doses of FS (mean  $\pm$  SE)

Material and dose ml <sup>1</sup>	Number of mice	Spleen weight mg	<sup>55</sup> Fe uptake, %		
			spleen	both femurs	erythrocytes
Saline 0.5 ml $\times$ 2	18	118 $\pm$ 11	8.7 $\pm$ 1.9	2.87 $\pm$ 0.2	3.9 $\pm$ 0.7
Sheep serum 0.25 $\times$ 2	20	128 $\pm$ 18	10.1 $\pm$ 1.9	2.58 $\pm$ 0.34	4.2 $\pm$ 0.9
0.50 $\times$ 2	20	169 $\pm$ 19	17.3 $\pm$ 2.1	2.01 $\pm$ 0.32	9.1 $\pm$ 1.1
1.00 $\times$ 2	18	171 $\pm$ 20	18.1 $\pm$ 3.3	2.02 $\pm$ 0.30	9.0 $\pm$ 1.2
0.25 $\times$ 4	16	149 $\pm$ 17	15.1 $\pm$ 1.8	2.00 $\pm$ 0.4	6.0 $\pm$ 0.9
0.25 $\times$ 4	20	170 $\pm$ 22	18.1 $\pm$ 4.0	1.87 $\pm$ 0.41	8.7 $\pm$ 1.1

Numbers in *italics* differ from control by *p* of 0.05 or less.

<sup>1</sup> Volume and number of daily injections.

## Results

The drop in hematocrit in normal mice following intravenous injection of 0.5 ml of absorbed FS was significantly smaller (1.3%) when compared to the drop caused by injection of nonabsorbed serum (2.9%). Table I shows the variation of the hematocrits at various intervals after the second injection of absorbed serum.

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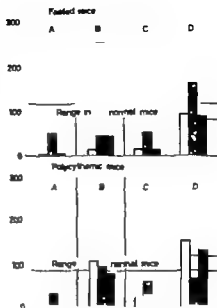


Fig. 1. Effects of FS on the erythropoiesis of fasted and polycythemic mice. Bars in the panels represent  $^{59}\text{Fe}$  uptake by the spleen (open bars), both femurs (skipped bars) and circulating reticulocytes (closed bars). A = Depressed untreated controls; B = response to erythropoietin alone; C = treatment with FS alone, and D = stimulation with erythropoietin after treatment with FS. Heights of bars represent values in each experimental condition and are expressed as percent of values in normal controls injected with isotonic saline solution which are taken as 100%.

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Table II. Effect of various doses of FS (mean  $\pm$  SE)

Material and dose ml <sup>1</sup>	Number of mice	Spleen weight mg	<sup>55</sup> Fe uptake, %		
			spleen	both femurs	erythrocytes
<i>Saline</i>					
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Numbers in italics differ from control by *p* of 0.05 or less.

1 Volume and number of daily injections.

hematopoietic progenitors and erythroid committed progenitors. This step of differentiation has been considered to be determined by conditions in the stromal and intercellular microenvironment or hematopoietic inductive microenvironment [1]. The mechanism of action of FS in the splenic responsiveness to EPO in the fasted mouse could then be interpreted as an activation of the local conditions of the hematopoietic inductive microenvironment to induce differentiation of HSC.

These results indicate that erythropoiesis can be influenced by non-EPO mechanisms and support the hypothesis of the existence of accessory factors controlling erythropoiesis. It also appears that the changes induced by FS in the splenic erythropoiesis of fasted mice can be a useful probe in studies of structure and functions of the erythropoietic inductive microenvironment.

# Acknowledgements

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Table III. FS activity after incubation with anti-EPO (mean  $\pm$  SE)

Material	<sup>55</sup> Fe incorporation, %		
	spleen	both femurs	erythrocytes
FS	16.9 $\pm$ 1.7	2.00 $\pm$ 0.36	8.6 $\pm$ 0.1
FS + anti-EPO	17.0 $\pm$ 1.9	2.01 $\pm$ 0.30	9.0 $\pm$ 1.1
Saline	9.1 $\pm$ 1.7	2.61 $\pm$ 0.18	4.0 $\pm$ 0.1
Saline + anti-EPO	10.2 $\pm$ 1.8	2.48 $\pm$ 0.29	4.1 $\pm$ 1.1

## Discussion

Our studies have demonstrated that the injection of FS in the mouse causes an increased rate of erythrocyte formation. The slight though consistent decrease of the hematocrit caused by FS in normal recipients raises the question of whether the change is due to a hemolytic effect of FS. The lack of correlation between the hematocrits and RBC <sup>55</sup>Fe uptake values in normal mice does not support such an interpretation. This can be taken as evidence against the possibility that the effects of FS may be mediated by a perturbation in oxygen exchange.

The erythropoietic depression in both plethoric and fasted mice has been ascribed to diminished EPO concentration [2-4]. The opposite pattern of response to EPO by the splenic and medullary fractions, however, suggests that fasting in addition to reducing EPO formation may alter erythropoiesis by some other mechanisms. In this regard it is worth noting that administration of FS restores the splenic responsiveness to EPO in the fasted mouse, bringing it closer to that of normal and plethoric mice on a normal diet.

Food deprivation does not modify the concentration of CFU in the spleen nor alter the effect of FS. In contrast, in the starved mouse the concentration of ESC is greatly reduced as evidenced by the erythropoietic response to a transient increase of endogenously released EPO. This may result from a reduction in the number of ESC in the spleen as a result of an interference in the pathway of transition between primitive

Table IV. Colony formation with FS (mean  $\pm$  SE)

Donors	Number of recipients	Number of colonies	Spleen weight
<i>Normal mice</i>			
None	19	14.6 $\pm$ 1.5	80.0 $\pm$ 3.0
FS (0.5 ml $\times$ 2)	18	24.0 $\pm$ 2.2	98.0 $\pm$ 3.1
<i>Fasted mice</i>			
None	17	13.6 $\pm$ 1.5	79.0 $\pm$ 2.2
FS (0.5 ml $\times$ 2)	17	22.0 $\pm$ 1.9	89.0 $\pm$ 2.4
	23 (controls) <sup>1</sup>		

Numbers in *italics* differ from those in the group that received cells from untreated donors by of 0.05 or less.

<sup>1</sup> Mice in this group were treated as the rest of the recipients but received no transplant.

EPO of the fasted mouse was reverted to normal (panel D). The effect of FS was not different after its incubation with anti EPO serum (table III).

Table IV shows the effects of FS on the number of CFU in the spleen as judged by spleen weight and number of colonies in the spleen 8 days after cell inoculation. Injection of FS caused a significant increase in the

## Case Reports

*Kindred B*

The proband was a 37-year-old Canadian female with chronic hemolytic anemia, slight icterus and splenomegaly. Her hemoglobins ranged around 9-10 g/dl, and reticulocytes varied between 4 and 11%. She did not require transfusions. Autohemolysis of sterile cells incubated for 48 h was increased to 9% (normal = <1%) and uncorrected by glucose (11.0%/4). Pronounced basophilic stippling was present in peripheral erythrocytes on the Wright's stained blood film. There was no history of consanguinity and precise ethnic lineage was unknown. No hematologic abnormalities were known to exist in her mother, 2 children, and 4 half-siblings. A detailed clinical summary of this patient has been presented previously [23].

*Kindred L*

The proband was a 47-year-old Canadian female of German heritage. As a child, she fatigued easily and was pale with slightly icteric sclerae. She was hospitalized twice at ages 23 and 24 because of anemia, for which she received several transfusions. At age 25, three-fold enlarged spleen was removed without perceptible clinical improvement. She received approximately 10 transfusions between ages 23 and 33 several during pregnancy. She has had symptoms referable to gallbladder disease with documented cholelithiasis and has continued to exhibit variable degrees of anemia with icterus, pallor and fatigability.

Her hemoglobin fluctuated around 10 g/dl with reticulocytes ranging between 13 and 26%. Coarse basophilic stippling on peripheral smears was pronounced, and there was moderate anisopoikilocytosis with the usual postsplenectomy alterations of erythrocyte morphology. The bone marrow exhibited moderate erythroid hyperplasia with increased iron stores and sideroblasts, but no megaloblastic features. Autohemolysis was increased to 6.2% correcting slightly to 4.1% in the presence of glucose. No hemoglobins or enzyme abnormalities were detectable by standard methods, and Coombs and Haptotests were normal. Erythrocyte osmotic fragility was slightly reduced.

There was no family history of consanguinity, anemia, blood dyscrasias, splenomegaly or gallbladder disease in parents, 4 siblings and 3 chil-

dren. The patient was born in Germany and emigrated to the US at age 36.

## Materials and Methods

Heparinized specimens of venous blood were air-exposed to Los Angeles under refrigeration for processing. Enzymes of oxidative and anaerobic glycolysis, glutathione and nucleotide metabolism and miscellaneous reactions were assayed according to *Beutler* [24] or as noted before [1]. Pyrimidine nucleotidase was assayed as described previously [1, 25]. Glutathione concentration was measured by the method of *Beutler et al.* [26]. Glycolytic intermediates were measured by the methods of *Milesami et al.* [27]. Extracts of whole blood or washed erythrocytes were deproteinized in perchloric acid or trichloroacetic acid for measurement of ultraviolet absorption spectra as outlined in an earlier report [1]. Details of chromatographic and electrophoretic identification of intracellular nucleotides were presented in the same report [1].

## Results

As in other cases of severe pyrimidine nucleotidase deficiency assays of a number of glycolytic and nonglycolytic erythrocyte enzymes showed only the elevated activities expected in populations with reticulocytosis and a young mean cell age. Distinct abnormalities were apparent in activities of pyrimidine nucleotidase and ribosephosphate pyrophosphokinase (RPF, PRPP synthetase, EC 2.7.6.1 table I), both of which are normally much more active in reticulocytes and young cells. When compared to appropriate controls, the mean nucleotidase activities in both probands were about 2-4% of expected values. Similarly RPF values were about 13 and 48% of expected in probands B and L, respectively.

The children of both probands, all of

## Additional Data from Two Kindreds with Genetically Induced Deficiencies of Erythrocyte Pyrimidine Nucleotidase

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**Key Words.** Anemia, hereditary hemolytic Enzyme deficiency Erythroenzymopathy Pyrimidine nucleotidase

**Abstract.** Two subjects, not previously reported in detail, had severe inherited deficiencies of erythrocyte pyrimidine nucleotidase. This was manifested hematologically by moderate hemolytic anemia with splenomegaly morphologically by punctate basophilic stippling of Wright's stained erythrocytes, and biochemically by intraerythrocytic accumulation of pyrimidine nucleotides, elevated concentrations of reduced glutathione, and partial deficiencies of ribosephosphate pyrophosphokinase. All 5 of their children were asymptomatic and phenotypically normal except for intermediate reductions in activities of pyrimidine nucleotidase consistent with heterozygosity for an autosomal recessive defect.

### Introduction

The syndrome caused by severe deficiency of erythrocyte pyrimidine nucleotidase (EC 3.1.3.5), transmitted as an autosomal recessive trait, has been observed in at least 27 individuals from 21 unrelated families [1-16]. Detection of so many cases in such a brief interval following its original description suggests that the gene frequency of this disorder may rival that of glucosephosphate isomerase [17] or even pyruvate kinase [18] deficiencies. Ready recognition may have been aided, however by a conspicuous morphologic hallmark, prominent basophilic stippling of Wright's stained erythrocytes. Further complex quantitative as-

says for nucleotidase activity are not necessary to establish the diagnosis, since simple ultraviolet absorption spectra of acid extracts of affected cells characteristically exhibit bathochromic shifts, reflecting the unique intracellular accumulation of pyrimidine nucleotides. The latter has only been observed clinically in severe inherited or lead-induced nucleotidase deficiencies [19-21].

The apparent frequency of this erythroenzymopathy encourages documentation of as many cases as possible to clarify the nature of the molecular defect(s) genetic predispositions, possible polymorphism, etc. This report records specifics of two cases that previously were mentioned only in footnotes [1].

Table II. Partitioning of ribonucleotides in proband erythrocytes from kindreds B and L

Subject	Total nucleotides $\mu\text{mol}/10^{10}$ RBC	Adenosine %	Inosine %	Uridine %	Cytidine %	Total pyrimidines %
B	7.4	10.3	8.5	36.5	44.7	81.2
L	6.1	9.2	9.9	32.6	48.3	80.9
Normal controls (n = 79)	1.5	97-100				< 1
Rebencloctosis (2.3-22.3%) controls (n = 15)	1.9	97-100				< 1

resulted from accumulations of nucleotides of cytidine and uridine that are found only in trace amounts in normal erythrocytes. The inosine component represents a deaminated degradation product of adenosine.

## Discussion

The syndrome induced by severe inherited deficiencies of erythrocyte pyrimidine nucleotidase is characterized by congenital hemolytic anemia with splenomegaly and erythrocytes with conspicuous basophilic stippling, elevated concentrations of glutathione, reduced RPK activities, and unique accumulations of intracellular pyrimidine nucleotides [1-28]. We have recently attempted to summarize the findings in diverse cases with this disorder [29], but much remains to be learned about genetic predisposition, polymorphism, pathophysiology and the nature of the underlying molecular defects. Currently available data indicate that higher incidences may be found in those of African, Jewish or peri-Mediterranean ancestry. The lack of historical evidence of consanguinity in at least half

of the known cases suggests that many of these are probably double heterozygotes for different defective genes at the pyrimidine nucleotide locus and that the gene frequency for these defects may be relatively high in the population at large.

It is apparent that the syndrome may result not only from absolute decreases in nucleotidase activities, but as well from molecular alterations producing aberrations in electrophoretic [2, 3, 5, 6, 10, 12] kinetic [2, 3, 5, 6] and thermostability characteristics [2, 3, 5, 6]. Unfortunately these specific features have not been studied in many instances, so their significance and frequency of occurrence remain uncertain. It becomes important to document such variations in phenotypic expression if the precise nature of these molecular defects is ever to be understood. To this end, it may be necessary to adopt internationally standardized methods to characterize nucleotidase abnormalities such as those recently proposed for pyruvate kinase isozymes [30]. Unlike the latter however the nucleotidase defects so far studied all seem to be associated with minimal residual activities, often less than 5% of values expected in comparably young



Table I. Biochemical abnormalities in kindreds B and L.

Subject	Pyrimidine nucleotidase $\mu\text{mol/min/g Hgb}$		Ribosephosphato pyrophosphokinase $\mu\text{mol/min}/10^{10}$ RBC	Glutathione $\mu\text{g}/10^{10}$ RBC	Ultraviolet absorption maximum nm
	UMP	CMP			
Kindred II					
Proband	0.6	0.3	6.9	1219	270
Daughter	3.3	2.7	34.8	644	257.5
Son	2.0	2.0	36.1	633	257.5
Mother			44.9		
Kindred L					
Proband	0.3	0.7	25.0	1146	270
Son	4.5	3.8	29.0	859	
Daughter	4.2	3.4	34.2	836	
Son	2.8	2.3	35.3	718	
Normal controls					
SD	7.3	6.2	30.5	666	257.5
	1.9	1.4	3.8	118	
Reticulocytosis controls*					
SD	15.1	12.2	51.6	655	257.5
	7.5	5.1		103	

Nucleotidase values were derived from 9 subjects with 2.4-36% reticulocytes. RPK value is mean from 10 subjects with 3-15% reticulocytes. GSH values were obtained from 18 subjects with 3.0-8.9% reticulocytes.

whom were obligate heterozygotes, had nucleotidase activities in the intermediate range, but RPK activities were essentially normal, supporting the postulate that partial deficiency of the latter is associated with severe nucleotidase deficiency only as an epiphenomenon [1]. Additional studies on cells from proband B indicated that residual RPK in hemolysates had normal Michaelis constants for ATP (0.03 mM) and for ribose 5-phosphate (0.11 mM) and a normal pH optimum (7.9).

Table I also records the elevated erythrocyte glutathione concentrations that have been noted uniformly in other instances of this syndrome. Again, an epiphenom-

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Both enzymatic and spectral assays indicated that total intracellular nucleotides were increased 4-4.5 times the concentrations usually found in normal controls. Adenine nucleotide concentrations are known to increase as a function of shortened mean cell age, but not nearly to the degree exhibited by these and other subjects with severe pyrimidine nucleotidase deficiency. Data in table II demonstrate that these elevations

Table II. Partitioning of ribonucleotides in proband erythrocytes from kindreds B and L

Subject	Total nucleotides $\mu\text{mol}/10^{10}$ RBC	Adenosine %	Inosine %	Uridine %	Cytidine %	Total pyrimidines %
B	7.4	10.3	8.5	36.5	44.7	81.2
L	6.1	9.2	9.9	32.6	48.3	80.9
Normal controls ( $n = 79$ )	1.5	97-100				< 1
Reticulocytosis (2.3-22.3%) controls ( $n = 15$ )	1.9	97-100				< 1

resulted from accumulations of nucleotides of cytidine and uridine that are found only in trace amounts in normal erythrocytes. The inosine component represents a deaminated degradation product of adenosine.

## Discussion

The syndrome induced by severe inherited deficiencies of erythrocyte pyrimidine nucleotidase is characterized by congenital hemolytic anemia with splenomegaly and erythrocytes with conspicuous basophilic stippling, elevated concentrations of glutathione, reduced RPK activities, and unique accumulations of intracellular pyrimidine nucleotides [1, 28]. We have recently attempted to summarize the findings in diverse cases with this disorder [29] but much remains to be learned about genetic predisposition, polymorphism, pathophysiology and the nature of the underlying molecular defects. Currently available data indicate that higher incidences may be found in those of African, Jewish or peri-Mediterranean ancestry. The lack of historical evidence of consanguinity in at least half

of the known cases suggests that many of these are probably double heterozygotes for different defective genes at the pyrimidine nucleotide locus and that the gene frequency for these defects may be relatively high in the population at large.

It is apparent that the syndrome may result not only from absolute decreases in nucleotidase activities, but as well from molecular alterations producing aberrations in electrophoretic [2, 3, 5, 6, 10, 12] kinetic [2, 3, 5, 6] and thermostability characteristics [2, 3, 5, 6]. Unfortunately these specific features have not been studied in many instances, so their significance and frequency of occurrence remain uncertain. It becomes important to document such variations in phenotypic expression if the precise nature of these molecular defects is ever to be understood. To this end, it may be necessary to adopt internationally standardized methods to characterize nucleotidase abnormalities such as those recently proposed for pyruvate kinase isozymes [30]. Unlike the latter however the nucleotidase defects so far studied all seem to be associated with minimal residual activities, often less than 5% of values expected in comparably young

Table I. Biochemical abnormalities in kindreds B and L.

Subject	Pyrimidine nucleotidase $\mu\text{mol/min/g Hgb}$		Ribosephosphate pyrophosphokinase $\mu\text{mol/min/10}^{10}$ RBC	Glutathione $\mu\text{g/10}^{10}$ RBC	Ultraviolet absorption maximum nm
	UMP	CMP			
Kindred B					
Proband	0.6	0.3	6.9	1219	270
Daughter	3.3	2.7	34.8	644	257.5
Son	2.0	2.0	36.1	633	257.5
Mother			44.9		
Kindred L					
Proband	0.3	0.7	25.0	1146	270
Son	4.5	3.8	29.0	859	
Daughter	4.2	3.4	34.2	836	
Son	2.8	2.3	35.3	718	
Normal controls					
SD	7.3	6.2	30.5	666	257.5
	1.9	1.4	3.8	118	
Reticulocytosis controls*					
SD	15.1	12.2	51.6	655	257.5
	7.5	5.1		103	

Nucleotidase values were derived from 9 subjects with 2.4-36% reticulocytes. RPK value is mean from 10 subjects with 3-15% reticulocytes. GSH values were obtained from 18 subjects with 3.0-8.9% reticulocytes.

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cells. It is even possible that such residual activities are not due to the aberrant isozyme but rather represent a normal minor isozyme that is only revealed by genetically induced absence of the major component [3].

Partial purification procedures, such as those developed by Torrance *et al.* [31] may therefore be necessary to concentrate sufficient nucleotidase activity to characterize adequately. Studies could doubtless be performed on preparations much less refined than the 250 000-fold purification achieved by Torrance *et al.*, and we [14] and others [6-32] have explored utilization of selected steps from their protocol, such as DEAE-cellulose or Sephadex fractionation and ammonium sulfate precipitation, to enhance low levels of residual activity. Such procedures, however, should be uniformly standardized among various laboratories if meaningful comparisons are to be made.

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## Human Haemoglobins and Haemoglobinopathies in Arabia. Hb O Arab in Saudi Arabia

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**Key Words.** Arabian peninsula Saudi Arabia Haemoglobinopathies Anthropology  
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**Abstract.** In the Arabian peninsula, haemoglobinopathies, though very interesting, are still far from being fairly established. With the exception of the eastern province of Saudi Arabia, where several aspects of the sickle-cell disease have been focused upon, few reports have appeared in the literature. However in 1974 one of us (M.A.F. E.H.) has initiated a comprehensive investigation, in collaboration with the Ministry of Health on haemoglobinopathies in various parts of the country. These studies have revealed a new pocket for the sickle-cell gene and a new structural abnormality. In the present report we describe the first finding of Hb O Arab in Saudi Arabia, which may shed more light on the anthropological significance of this haemoglobin variant.

### Introduction

Hb O Arab is a relatively rare abnormal haemoglobin which has been found in a specific geographical distribution. It has been found mostly in families in Sinai [31] Mainland Egypt [4, 18] Sudan [17, 38] Southern Yemen [4] Yugoslavia [12] and Bulgaria [19]. Hb O Arab has also been found in American Negro families [2] Greek families [34] and Jamaicans [25, 28]. The highest incidence being reported in families from Bulgaria [20] and Yugoslavia [12, 13].

It has been suggested that Hb O Arab could be used as an anthropological marker specific for Arab ethnic groups [4]. Others

have suggested that the abnormal haemoglobin could originate from a non-Arab population, probably related to pre-semitic Egyptians [38] or Pariah tribes of Northern India [13]. An alternative proposal was that Hb O Arab possibly originated independently from Bulgaria [20]. The Arabic origin of Hb O Arab has been less advocated because of the lack of its discovery in the Arabian peninsula [13, 24].

In the present study we report our findings in a Saudi Arabian female heterozygote for Hb O Arab. Structural studies and biosynthesis showed the patient to be doubly heterozygous for Hb O Arab and thalassaemia.

## Materials and Methods

The proband was a 38-year-old female admitted to the Central Hospital of Riyadh. Initially she complained of epigastric pain and suffered from anaemia; she was unresponsive to iron therapy and had splenomegaly. She came from an area North of Riyadh and none of her family was available for investigation.

Haematological indices were determined using standard methods [11] and iron was determined following Seefelt's method [37]. Haemolysates

was prepared and examined for the presence of haemoglobin variants by electrophoresis on agarose gel using barbital buffer pH 8.6 [10]. Minor haemoglobin constituents, HbA<sub>2</sub> and F were determined by an elution method following cellulose-acetate electrophoresis [23, 26] and alkaline denaturation [6], respectively. Preliminary characterization of structural abnormality and differentiation between Hb C, E and O Arab were carried out by agar gel electrophoresis [32]. Structural studies were performed on the ammoniolyzed [9, 30] abnormal  $\beta$ -chains following fractionation of globin by ion-exchange chromatography [7]. Identification of the abnormal haemoglobin was confirmed by finger-printing [35] and amino acid analysis [36] following enzymatic degradation of the abnormal aminoacylated  $\beta$ -chain.

*In vivo* biosynthesis was carried out by the method of Luzzati and Borzok [25] as modified by us [15]. Thereafter globin was prepared [33]

from whole-cell lysate or purified haemoglobin solution following fractionation on Sephadex G-100 column [8]. Globin was then separated into its constituents on CM 23 cellulose column [7]. Incorporation of <sup>3</sup>H-leucine into globin chains was measured as total cpm/chain, using the Tracerlab cori Matic 200 and as specific activity as presented as cpm/fraction absorbance at 280 nm, measured on a Zeiss recording spectrophotometer (DMR 21). When haemoglobin solution was used, the specific activity was determined as dpm per fraction/optical density unit at 540 or 514 nm.

## Results

Laboratory tests revealed hypochromic microcytic cells with a haemoglobin concentration of 8.3 g/dl, packed cell volume of 0.26 litres/litre, a red blood cell count of  $4.2 \times 10^{12}/l$  and a reticulocyte count of 5%. Marked anisocytosis and poikilocytosis and occasional target cells were observed. Iron determination indicated a mild iron deficiency of 13  $\mu$ mol/l compatible with the microcytic hypochromic cells observed in the peripheral blood.

Hb A<sub>2</sub> amounted to 3.7%, Hb F to 2.6% and the abnormal variant up to 42% of the

Table 1. Globin chain biosynthesis in peripheral blood from Hb O Arab heterozygote

Globin source	Incorporation of <sup>3</sup> H-leucine into globin chains											
	Globin chain extract, without haemin in incubation						Globin chain extract, with haemin in incubation					
	total radioactivity cpm/chain			specific activity cpm/280 nm			total radioactivity cpm/chain			specific activity cpm/280 nm		
	$\overline{\beta^*}$	$\overline{\beta^*}$	$\overline{\beta}$	$\overline{\beta^*}$	$\overline{\beta^*}$	$\overline{\beta}$	$\overline{\beta^*}$	$\overline{\beta^*}$	$\overline{\beta}$	$\overline{\beta^*}$	$\overline{\beta^*}$	$\overline{\beta^*}$
Whole cell	0.51	0.47	0.92	0.55	0.51	0.93	0.60	0.50	0.83	0.62	0.51	0.82
Purified tetramer	0.29	0.43	1.05	0.32	0.43	1.04	0.45	0.41	0.91	0.42	0.45	1.07

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### Introduction

Hb O Arab is a relatively rare abnormal haemoglobin which has been found in a specific geographical distribution. It has been found mostly in families in Sinai [31] Mainland Egypt [4, 18] Sudan [17, 38] Southern Yemen [4] Yugoslavia [12] and Bulgaria [19]. Hb O Arab has also been found in American Negro families [2] Greek families [34] and Jamaicans [25, 28]. The highest incidence being reported in families from Bulgaria [20] and Yugoslavia [12, 13].

It has been suggested that Hb O Arab could be used as an anthropological marker specific for Arab ethnic groups [4]. Others

have suggested that the abnormal haemoglobin could originate from a non-Arab population, probably related to pre-~~semi~~ Egyptians [38] or Pariah tribes of Northern India [13]. An alternative proposal was that Hb O Arab possibly originated independently from Bulgaria [20]. The Arabic origin of Hb O Arab has been less advocated because of the lack of its discovery in the Arabian peninsula [13, 24].

In the present study we report our findings in a Saudi Arabian female heterozygote for Hb O Arab. Structural studies and biosynthesis showed the patient to be doubly heterozygous for Hb O Arab and thalassemia.

defects which may induce haemolytic anaemia, as a result of membrane damage due to structural alteration in the amino acid sequence or an imbalance in the haemoglobin subunit production. The effect of amino acid substitution on the properties of the haemoglobin molecule is variable and so are the clinical manifestation and severity of the individual haemoglobinopathies.

The haemoglobinopathies of structural origin, which may result in anaemia, can be due to the presence of sickle-cell gene homozygosity or unstable haemoglobins. Double heterozygosity for two abnormal haemoglobins or structural and biosynthesis abnormalities can also be pathologic.

In general, haemoglobin structural disorders which may result in clinical manifestations are due to the presence of the most common homozygous haemoglobinopathies, e.g. Hb<sub>s</sub>, S, E and C diseases. With the exceptions of these abnormal haemoglobins, few homozygous states have been reported. A homozygote for Hb G Philadelphia was reported [27] and a case of Hb K Woolwich has also been found [2]. There are few reported cases of Hb Tongariki [1-5], Hb Acra [23] identified as Korie Bu, Punjab [29] and Hb G Szabu [21] none of which, in homozygotes, seem by themselves to give rise to haematological abnormalities.

Hb O Arab has been found in a homozygous carrier and in combination with Hb S [3, 17, 28] and Hb C [28] and  $\beta^0$  and  $\beta^+$ -thalassaemias [19, 20]. Hb O Arab thus reported appear to cause a mild clinical manifestation in the homozygous states and in combination with Hb S when associated with  $\beta^0$ -thalassaemia, the abnormal haemoglobin results in a severe clinical condition, comparable to that of Hb E-thalassaemia. However in combination with  $\beta^+$ -thalassaemia

and in the heterozygous states, the carrier is symptomless [20, 28].

The present work describes a patient with the combination Hb O Arab/ $\alpha$ -thalassaemia who has undergone splenectomy during the present study. She was suffering from haemolytic anaemia which could explain the favoured survival of  $\gamma$ - and  $\delta$ -chain resulting in an increase in Hbs A<sub>2</sub> and F proportions compared to Hb A. However her  $\alpha$ -thalassaemia was evident as addition of haemin did not correct the marked decrease in  $\alpha$ -chain biosynthesis.

The present paper recording the finding of Hb O Arab in the centre of the Arabian peninsula links its previous discovery in Aden in the south and in Northern Yemen. The discovery of Hb O Arab in Saudi Arabia may indicate that the recent cases of Hb C detected in the east of the country using paper electrophoresis as the only means of identification [16] may in fact have been cases of Hb O Arab.

One can only stress that if haemoglobin variants are to be used as anthropological markers, they must be definitely identified and the racial origins of the propoiti stated clearly.

### Acknowledgement

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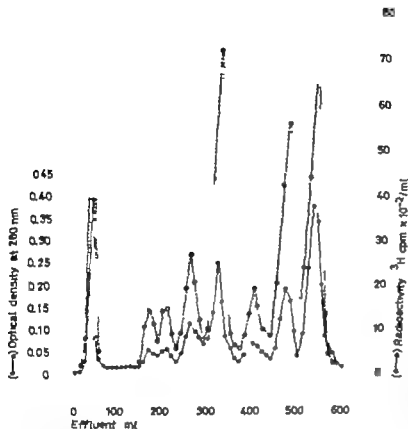


Fig. 1 Elution profile of globin chains from Hb O Arab heterozygote. The peaks shown on the figure represent non-globin proteins,  $\gamma$ ,  $\beta$ ,  $\delta$  and  $\epsilon$  chains, respectively.

total haemoglobin concentration the remainder being Hb A. On the agarose film, at pH 8.6 the variant migrated with Hb A<sub>2</sub>, but in agar gel electrophoresis, at pH 6.0 the abnormal haemoglobin moved toward the anode in a position identical to that of Hb O Arab. Furthermore, the haemoglobin variant was identified, in the amino acid analysis, as Hb O Arab with the substitution  $\beta_{101} \text{ Glu} \rightarrow \text{Lys}$  as reported previously [3].

*In vitro* biosynthesis in the patient's reticulocytes, performed in haemin-free and haemin-containing media, showed a marked decrease in the  $\alpha$ -chain biosynthesis compared with that of non- $\alpha$ -chains (table I). The ratio of  $^3\text{H}$  leucine incorporated into  $\alpha$ - and non- $\alpha$ -chains indicated, on globin fractionation (fig. 1), a moderate stimulation of  $\alpha$  over non- $\alpha$  in globin prepared from whole-

cell lysate. Gel filtration revealed no sizeable chain dimer. The preferential haemin effect on  $\alpha$ -chain biosynthesis in purified tetramer molecules was higher than that observed in whole-cell globin. However, there was a substantial general loss of  $\alpha$ -chain radioactivity on purification of haemoglobin tetramer in the Sephadex G-100 column, though the loss was greater when biosynthesis was carried out in the absence of haemin. Similar results were also observed in some iron-deficient cases in our laboratory.

## Discussion

Anaemia due to haemoglobin disorders is usually classified under intracorpuscular

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### Case Report

Hematologic studies done in the course of a routine evaluation of 56-year-old woman demonstrated the presence of numerous target cell forms in a stained smear of her peripheral blood. Her hematologic values were hemoglobin 14.6 g/dl, RBC  $4.94 \times 10^6/\text{ml}$ , MCV 93 fl, MCH 30.1 pg, and reticulocyte count 1.4%. Her physical examination showed no abnormality.

### Results

Hemoglobin electrophoresis in starch gel at pH 8.6 demonstrated two major hemoglobins to be present in addition to Hb A. These included a slowly migrating hemoglobin with the electrophoretic mobility of Hb A<sub>2</sub> and a rapidly moving hemoglobin with mobility slightly greater than that of Hb N Seattle (fig. 1). The slowly migrating hemo-

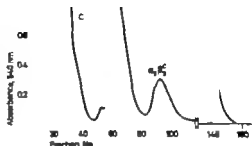


Fig. 2. Fractionation of hemoglobins from the patient by DEAE-cellulose chromatography. The hemoglobins were eluted with glycine-containing buffers as described by Abraham *et al.* [1]. Mean recovery values from three chromatographic separations were: Hb C  $31.0 \pm 1.0\%$ , Hb A  $44.3 \pm 0.6\%$ , the hybrid hemoglobin  $12.3 \pm 0.2\%$ , and Hb I  $12.4 \pm 0.3\%$ .

globin had the characteristic electrophoretic mobility of Hb C in agar gel at pH 6.2.

Hemoglobin fractionation by DEAE-cellulose chromatography (fig. 2) allowed the slowly and rapidly migrating hemoglobins to be isolated in pure form. An additional hemoglobin with electrophoretic mobility identical to that of Hb A was also resolved by the chromatography and had an elution position immediately after that of Hb A (fig. 2). The globin chains of each hemoglobin were separated by CM-cellulose chromatography and the amino acid composition of each was determined.

The identity of the earliest emerging hemoglobin as Hb C was based on its electrophoretic and chromatographic behavior as well as the properties of its globin chains and no further characterization was carried out.

Chromatography of globin chains from the last emerging hemoglobin demonstrated the presence of  $\beta$  as well as an  $\alpha$ -chain having an elution position considerably ahead

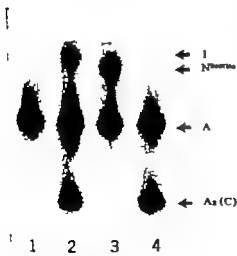


Fig. 1. Starch gel electrophoresis at pH 8.6. 1 = Normal control (AA); 2 = patient; 3 = heterozygote for Hb N Seattle ( $\beta$  61 lys  $\rightarrow$  glu), and 4 = Hb C trait.

## Hemoglobin C-I-A

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**Key Words.** Globin structural variants Hybrid hemoglobin

**Abstract** Hemoglobin I ( $\alpha 16 \text{ lys} \rightarrow \text{glu}$ ) was identified in association with hemoglobins C and A in a black American woman who had no clinical or hematologic disease. Her erythrocytes contained four major hemoglobins, all of which were separable by DEAE-cellulose column chromatography. Nearly 50% of the total recovered  $\alpha$ -chains were present in combination with I, suggesting that there may have been preferential formation of the  $\alpha 16 \text{ lys} \rightarrow \text{glu}$  hybrid hemoglobin.

Hemoglobin I ( $\alpha 16 \text{ lys} \rightarrow \text{glu}$ ) was first described in 1955 by Rucknagel *et al* [11] who discovered this variant in a black American family. Subsequent reports have described the identification of Hb I in 11 additional families from widely separated areas of the world [4]. In this communication we describe the hematologic and laboratory findings of an American woman who was found to have Hb I as well as Hb C, and who thereby provided an opportunity to examine the interaction of these abnormal hemoglobins.

### Methods

Hematologic measurements were made with a Coulter model S cell counter which was standardized daily using a commercial standard. Stroma-free hemolysates were prepared from washed

erythrocytes after addition of an equal volume of water and 0.5 vol carbon tetrachloride. Hemoglobin electrophoresis was carried in starch gel with Tris-EDTA borate buffer pH 8.6 [13] and in agar gel with citrate buffer at pH 6.2 [7]. For separation and quantitation of the various hemoglobins, DEAE-cellulose column chromatography was performed using glycine-containing buffers as described by Abraham *et al* [1]. Globin chain chromatography was carried out according to the procedure of Clegg *et al* [3].

The purified  $\alpha$ -chain was digested with trypsin and the resulting digest subjected to peptide mapping [3]. The abnormal peptide was isolated from a trypsin digest by gel filtration using a  $240 \times 2.5$  cm column of Sephadex G-25 which was equilibrated with 20% acetic acid. Traces of contaminating peptides were removed by chromatography on a column of PA 35 using a linear pyridine-acetic acid gradient [6]. Globin chains and peptides for amino acid analysis were hydrolyzed in 6 N HCl at  $110^\circ\text{C}$  for 24 h. The analyses were made with a Beckman-Spinco model 121 B1 amino acid analyzer equipped with a system AA integrator.

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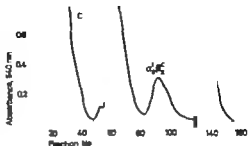


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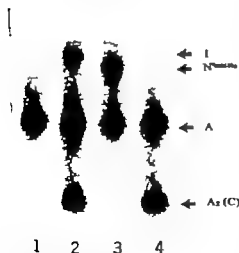


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of that of  $\alpha^A$ . A peptide map of tryptic peptides from the variant  $\alpha$ -chain showed  $\alpha$ -T 3 and T-4 to be missing, with the presence of an abnormal peptide which gave positive staining reactions for tryptophan, arginine, tyrosine and histidine. The amino acid composition of the purified peptide (table I) indicated the presence of a lys  $\rightarrow$  glu substitution at  $\alpha$  16 demonstrating therefore, that the variant globin chain was  $\alpha^I$ .

The hemoglobin fraction that eluted from the DEAE-cellulose column immediately following Hb A contained mainly  $\alpha^I$  and  $\beta^C$ -chains (fig 3). A very small quantity of  $\beta^A$ -chains was also present and possibly some  $\delta$ -chains as well, but  $\alpha^I$  was the only  $\alpha$ -chain detected. This hybrid hemoglobin fraction represented  $12.3 \pm (SD) 0.2\%$  of the total based on three DEAE-cellulose chromatographic determinations. Hb I the other  $\alpha^I$ -containing fraction made up  $12.4 \pm 0.3\%$ .

Table I. Amino acid composition of the  $\alpha$ -T 3-4 peptide from the variant  $\alpha$ -chain<sup>1</sup>

Amino acid	Normal composition of $\alpha$ -T 3-4	Composition of the variant $\alpha$ -T 3-4 peptide
Lysine	1	0 -1
Histidine	1	1.02
Arginine	1	0.90
Glutamic acid	3	4.07 +1
Glycine	4	3.94
Alanine	6	6.09
Valine	1	0.97
Leucine	1	1.09
Tyrosine	1	0.92
Tryptophan <sup>2</sup>	1	+

<sup>1</sup> Values represent the number of amino acid residues per peptide.

<sup>2</sup> Tryptophan was detected by spot testing on paper

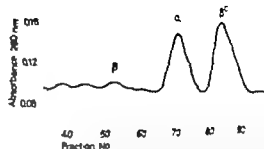


Fig. 3. Globin chain separation from the hemoglobin hybrid. CM-cellulose chromatography was performed with buffers containing 8M urea according to Clegg et al. [3].

## Discussion

Previous reports have described twelve different hemoglobin combinations in which  $\alpha$ -chain structural variants were present together with  $\beta$ -chain variants, with the formation of hybrid hemoglobins [2, 5, 9, 12, 14]. In each of these combinations the quantitative distribution of the normal, variant, and hybrid hemoglobin forms fell within the expected ranges, based on the relative quantities of each of the globin chains present and assuming random association of  $\alpha$ - and non- $\alpha$ -globin chains in the formation of the hemoglobin tetramer molecules.

In the individual described in this report a disproportionate fraction of  $\alpha^I$ -chains appears to have been present in combination with  $\beta^C$  whereas  $\beta^A$  (+  $\delta$ ) accounted for 43.4% of the patient's non- $\alpha$ -chains, the  $\alpha^I/\beta^C$  hybrid represented 49.8% of the recovered  $\alpha^I$ -chains, suggesting that there may have been preferential formation of the hybrid hemoglobin. This pattern of  $\alpha$ - and  $\beta$ -chain association *in vivo* also contrasts interestingly with previous observations that were made in experiments using artificial mixtures of Hb C and Hb I [10]. Under conditions that promoted globin chain by

bridization *in vitro*, formation of the  $\alpha^1\beta^1$  hybrid was observed not to proceed to the theoretical equilibrium fraction, even over very long periods of time [10]

Hb I has previously been shown to have normal functional properties [8] consistent with the hematologic findings of this patient, which were indistinguishable from those of individuals having Hb C trait.

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## Recurrent Thrombosis in a Patient with Factor XII Deficiency

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**Abstract.** The case history of a patient with moderate factor XII deficiency and recurrent deep vein thrombosis is described. A decreased resting fibrinolytic capacity suggests that *in vivo* Hageman factor acts mainly as a promotor of clot dissolution. It further indicates that the *in vitro* demonstration of factor XII as an activator for other biochemical pathways might be of minor importance *in vivo* as alternative pathways for activation of these systems exist.

### Introduction

Although factor XII (Hageman factor) is an activator of several distinct biochemical pathways, the intrinsic pathway of coagulation the kallikrein-kinin system the plasminogen mediated fibrinolysis and the activation of the first component in the complement system the relationship of factor XII and the pathways in which it is involved is only partly clarified [12]. The relative significance of the function of factor XII *in vivo* in these processes is illustrated by the lack of bleeding tendency in patients with Hageman trait, whereas thrombotic episodes have been reported [2, 10, 13]. This report describes a further patient with a low

factor XII level in whom recurrent venous thrombotic episodes occurred

### Case Report

At the time of investigation the patient was a 36-year-old male. There was no previous history of a bleeding tendency either in the patient or his family. At the age of 7 he had a fracture of the right forearm without complications. 4 years prior to the present investigation he developed a deep thrombosis in the right leg and phlebography demonstrated thrombosis of the popliteal vein extending to the right external iliac vein. He was treated with coumarin anticoagulants and was able to continue his job as a foundry worker. Except for a short period of swelling of the right leg, no further thrombotic episodes occurred until he

stopped the anticoagulant therapy 4 months prior to the present investigation. 2 months later the left leg became swollen and painful. After 4 weeks he was admitted to hospital and anticoagulation therapy was started again. The swelling of the leg persisted and the patient was transferred to this hospital for further treatment. Phlebography of the left leg revealed an almost complete blockage of the deep veins in the leg and thigh with numerous superficial collateral veins.

Physical examination revealed an oedematous left leg and foot with slight cyanosis of the toes.

He was treated with properly adjusted coumarin in anticoagulation therapy and elevation of the leg and after 3 weeks the swellings disappeared leaving only slight discoloration of the skin of the foot.

Routine examinations were (reference values in parentheses) leucocyte count  $4.1 \times 10^9/\text{liter}$  (4.0–11.0) with normal white cell distribution, haemoglobin 9.0 mmol/l (8.8–10.6), sedimentation rate 36 mm/h, serum proteins 74 g/l (69–83), serum creatinine 75  $\mu\text{mol/l}$  (63–127), urine examination for blood, glucose and proteins, nil. Examination of the complement system, normal values for C3, C4, C1 esterase inhibitor C3 and C4. The  $\alpha$ -1-antitrypsin concentration was 3.9 g/l (1.7–3.2) and  $\alpha$ -2-macroglobulin 2.3 g/l (1.3–3.9). Serum total lipids 4.8 g/l (4.0–7.5), cholesterol 4.1 mmol/l (3.3–6.7), triglycerides 0.73 mmol/l (0.35–1.75),  $\beta$ -lipoproteins 2.8 g/l (0–6.0), pre  $\beta$ -lipoproteins 0.9 g/l (0.2–2.0) and  $\alpha$ -lipoproteins 3.2 g/l (1.6–5.0).

### Material and Methods

Blood for coagulation studies was obtained by venipuncture and collected in plastic tubes after discarding the first 2 ml of blood. Sodium citrate 3.8% w/v 1–9 parts of blood were used as anticoagulant. Immediately after sampling, blood was centrifuged for 10 min at 1,500 g and plasma kept on ice until analysis took place within 1 h after sampling. In cases of later analysis plasma was immediately frozen and kept at  $-60^\circ\text{C}$ . Platelet-rich plasma for platelet examination was obtained after centrifuging the blood for 5 min at 200 g. Platelet-rich plasma was kept at room temperature until analysis within 2 h after

sampling. Activated partial thromboplastin times (APTT) were determined with Carptest® reagent from Nyco-Med, Oslo, Norway. Prothrombin time with tissue thromboplastin and bovine serum, both supplied by Statens Seruminstitut, Copenhagen, Denmark. Thrombin time was determined after adding 1 part of thrombin (Thrombin Leo® Løvens Kemiske Fabrikker Ballerup, Denmark), corresponding to 1 U to 2 parts of plasma. Fibrinogen content was measured as clottable protein by the technique of Claessens [4]. Factor levels were measured using one-stage assay with commercial hypofibrinized factor-deficient plasmas and control plasmas supplied by Mertz and Dade AG Bernex, Switzerland.

Antithrombin-III and plasminogen concentrations were measured by the radial immunodiffusion technique with M-Parigen plates® from Behringwerke AG, Marburg, FRG using own reference values and control. Antithrombin-III activity was determined with 'Coatest, Antithrombin-III' from KabiVitrum, Stockholm, Sweden.

Enoglobulin clot lysis time was performed according to the method of Astrup and Rasmussen [1]. Fibrinogen degradation products were determined with semiquantitative modification of latex fixation test (Thrombo-Weico-test supplied by Wellcome Research Laboratories, Beckenham, England).

Factor VIII antigen level was determined by Laurell octet immunoelectrophoresis using factor VIII antibody from Behringwerke, 200  $\mu\text{l}$  in 24 ml of agarose gel, 10 g/l in barbital buffer pH 8.4. The electrophoresis was carried out at 2 V/cm for 20 h.

The coagulation studies and the platelet aggregation examinations were performed on a semi-automated coagulation apparatus 'Fibromat' from Bae and Berntsen, Copenhagen in 300- $\mu\text{l}$  cylindrical cuvette thermostated at  $37^\circ\text{C}$  using magnetic stirring at 900 rpm for platelet aggregation studies. Platelet adhesivity was determined according to the method of Hellm [7].

### Results

On admission to this hospital the patient's haemostatic profile (table I) revealed



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associate with an enhanced thrombosis tendency. The normal bleeding time, however, indicates the presence of a bypass mechanism *in vivo*. The elevated factor VIII level should not be considered as causing a hypercoagulable state since factor VIII is an acute phase reactant [8] showing high values in many disease states without causing an increased clotting tendency [3, 11]. The patient's factor VIII level was checked 8 months after the acute episode and was still of the same magnitude.

The patient's antithrombin III level, as measured by an immunological assay, was high ruling out an antithrombin-III deficiency. This was further verified by the normal level of antithrombin-III measured by the euglycine activity test. In spite of normal plasminogen level, the patient clearly showed a reduction in intravascular fibrinolytic capacity characterized by a markedly prolonged euglobulin clot lysis time. We think that this finding reflects a decreased Hageman factor dependent activation of fibrinolysis. Fibrinolysis, however, could be activated by vascular activator as demonstrated by the normalisation of the euglobulin clot lysis time after venous stasis. The hypothesis that deficiency of factor XII may be the cause of the patient's clinically increased thrombosis tendency cannot be proven until further investigation clarifies the relative *in vivo* significance of the interactions of the biochemical pathways in which the Hageman factor participates. There is evidence for the existence of alternative pathways for activation of some of these systems. Factor XI might be directly activated on platelets exposed to collagen [16]. Kinin-mediated inflammation seems to occur in patients with the Hageman trait [5] and activation of the complement cascade can be initiated both

by the alternative pathway and immune complexes [6, 17]. Comparison between our patient and the case reported by McPherson [10] seems to indicate that Hageman factor may have considerable importance in normal fibrinolysis.

We feel this case report of a patient with moderately decreased factor XII level and recurrent thrombosis focuses on the *in vivo* function of factor XII and in some respects parallels the documented enhanced thrombosis tendency in patients with moderate decreased antithrombin III levels [9].

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Table I. Haemostatic profile of the patient when first seen at the Coagulation Laboratory

	Patient	Reference values
Platelet count/l	$141 \times 10^9$	$140-340 \times 10^9$
Platelet adhesiveness, relative	0.03	0.17-0.34
Thrombin time, sec	13	13-17
APTT sec	99	25-40
Prothrombin time relative	0.33	0.85-1.15
Fibrinogen, $\mu\text{mol/l}$	13	5-12
Bleeding time Ivy min	6	3-8
Factor VIII activity relative	3.25	0.60-1.60
Factor VIII antigen, relative	3.18	0.60-1.75
Factor IX activity relative	0.60	0.60-1.40
Factor XI activity relative	1.50	0.60-1.60
Factor XII activity relative	0.24	0.60-1.60
Antithrombin-III antigen, g/l	0.41	0.24-0.37
Antithrombin-III activity relative	1.36	0.85-1.30
Plasminogen, mg/l	138	85-140
Euglobulin clot lysis time, h	>12	1-4
Euglobulin clot lysis time after venous stasis, min	15-30	<60
Fibrinogen degradation products, mg/l	0	0-10
Platelet aggregation studies		
2nd phase aggregation after ADP stimulation, $\mu\text{mol ADP/l}$	40	1-4
2nd phase aggregation after ristocetin stimulation, g/l	11	0.5-1.6

a definite but not severe deficiency of factor XII which was detected on the basis of a markedly prolonged APTT

Other factors in the intrinsic coagulation cascade were 60% or more compared with control plasma. A circulating anticoagulant could be ruled out, as a mixture of equal volumes of control and patient plasma yielded a normal APTT. The euglobulin clot lysis time could be shortened after venous stasis at 80 mm Hg for 5 min, indicating a normal vascular plasminogen activator release.

The haemostatic profile was reinvestigated 8 months later in a period when the patient was free of any acute illness. Nearly exactly the same results were obtained, especially concerning factor XII level (0.25).

The low prothrombin time and the marginal factor IX level was due to anticoagulant therapy which for obvious reasons could not be interrupted.

## Discussion

In spite of the patient's factor XII deficiency and the prolonged APTT he developed a severe deep venous thrombosis in both legs. Investigation of the platelet aggregation ability did not reveal any increased aggregation tendency. As has been demonstrated earlier in patients with Hageman trait [14, 15] platelet adhesiveness on glass surfaces was very low. This finding, apart from being unexplained, is difficult to

## Short Communications

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### Acute Leukaemia after Prolonged Chlorambucil Treatment for Non-Malignant Disease Report of a New Case and Literature Survey

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**Key Words:** Chlorambucil Acute leukaemia Immunosuppressive agents

**Abstract.** A case of acute myeloblastic leukaemia is reported in a patient treated with chlorambucil during 72 months for multiple sclerosis. In a review of the medical literature, 43 additional reports of acute leukaemia after chlorambucil therapy for non-malignant diseases were found. Most cases are of a non-lymphoid type and preceded by various blood and bone marrow abnormalities. The prognosis is poor with a short survival. The risk of developing acute leukaemia after long-term immunosuppressive treatment with chlorambucil is emphasized.

Chlorambucil, an alkylating agent with immunosuppressive properties, has been used for the treatment of a wide spectrum of non-malignant conditions. Its leukaemogenic action on such patients has been recently emphasized [4]. We report a new case of acute myeloid leukaemia in a patient treated with chlorambucil for multiple sclerosis.

#### Case Report

A 20-year-old woman was first evaluated in 1960 for recent history of diplopia and rapid loss of vision in the right eye. The diagnosis of multiple sclerosis was in little doubt and treatment was started with synthetic corticosteroids given intrathecally and intravenously. In December 1969 the patient developed rapidly progressive weakness of the right leg and she was placed

on chlorambucil, 8 mg daily. Frequent hematologic controls failed to reveal cytopenia but chlorambucil was discontinued in 1976. The cumulative dose was about 10 g during an overall period of 72 months.

From 1976 to 1978, the patient frequently experienced episodes of fatigue, fever and urinary infection. In October 1978, macrocytic anaemia occurred with thrombocytopenia. Leukopenia with granulocytopenia occurred in November 1978.

The patient was referred to us, in January 1979 because of pancytopenia (Hb = 9.9 g/dl, WBC =  $3.3 \times 10^9/l$ , platelets =  $50 \times 10^9/l$ ). Physical examination showed skin pallor without jaundice. There was no enlargement of the spleen, liver or lymph nodes. The bone marrow sternal aspirate displayed normal cellularity, megaloblastoid changes of the erythroid series and abnormal myeloid maturation with increased percentage of myeloblasts (18%). Asar rods were not present. The reaction for myeloperoxidase was positive. The reaction for non-specific esterase was positive,

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### Acute Leukaemia after Prolonged Chlorambucil Treatment for Non-Malignant Disease Report of a New Case and Literature Survey

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**Key Words.** Chlorambucil. Acute leukaemia. Immunosuppressive agents

**Abstract.** A case of acute myeloblastic leukaemia is reported in a patient treated with chlorambucil during 72 months for multiple sclerosis. In a review of the medical literature, 43 additional reports of acute leukaemia after chlorambucil therapy for non-malignant diseases were found. Most cases are of a non-lymphoid type and preceded by various blood and bone marrow abnormalities. The prognosis is poor with a short survival. The risk of developing acute leukaemia after long-term immunosuppressive treatment with chlorambucil is emphasized.

Chlorambucil, an alkylating agent with immunosuppressive properties, has been used for the treatment of a wide spectrum of non-malignant conditions. Its leukaemogenic action on such patients has been recently emphasized [4]. We report a new case of acute myeloid leukaemia in a patient treated with chlorambucil for multiple sclerosis.

#### Case Report

A 20-year-old woman was first evaluated in 1960 for a recent history of diplopia and rapid loss of vision in the right eye. The diagnosis of multiple sclerosis was in little doubt and treatment was started with synthetic corticotrophin given intrathecally and intravenously. In December 1969 the patient developed rapidly progressive weakness of the right leg and she was placed

on chlorambucil, 6 mg daily. Frequent haematologic controls failed to reveal cytopenia but chlorambucil was discontinued in 1976. The cumulative dose was about 10 g during an overall period of 72 months.

From 1976 to 1978, the patient frequently experienced episodes of fatigue, fever and urinary infection. In October 1978, macrocytic anaemia occurred with thrombocytopenia. Leukopenia with granulocytopenia occurred in November 1978.

The patient was referred to us, in January 1979 because of pancytopenia (Hb = 9.9 g/dl, WBC =  $3.3 \times 10^9/l$ , platelets =  $50 \times 10^9/l$ ). Physical examination showed skin pallor without jaundice. There was no enlargement of the spleen, liver or lymph nodes. The bone marrow aspirate displayed normal cellularity megaloblastoid changes of the erythroid series and abnormal myeloid maturation with increased percentage of myeloblasts (18%). Auer rods were not present. The reaction for myeloperoxidase was positive. The reaction for non-specific esterase was positive,



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unaffected by exposure to sodium fluoride. No ringed sideroblast was seen with the Perl's staining. Bone marrow culture in agar using the technique of Pike and Robinson showed a normal pattern of growth. Bone marrow cells karyotype was not performed. The patient was discharged without any treatment.

A low percentage of myeloblasts on blood counts appeared in February 1979. The bone marrow myeloblasts count rose to 45% in March 1979. The diagnosis of acute myeloid leukaemia was established, M1 type according to the FAB group classification [1]. Intensive chemotherapy was started with vincristine, cytosine arabinoside and daunorubicin. This treatment resulted in severe marrow aplasia leading to a rapid fatal outcome.

## Discussion

Chlorambucil has been widely used for immunosuppression in various autoimmune or inflammatory diseases. We were able to collect, mainly from the references indexed in the *Index Medicus* of the National Library of Medicine, 43 additional cases of acute leukaemia occurring after prolonged chlorambucil treatment for non neoplastic diseases [2-14]. Among the 44 patients there were 10 males, 33 females and 1 patient for whom sex was not specified. The underlying diseases included rheumatoid arthritis (18 cases), glomerulonephritis and nephrotic syndrome (8 cases), multiple sclerosis (8 cases), psoriatic arthritis (4 cases), Wegener's granulomatosis (2 cases), progressive systemic sclerosis (1 case), juvenile rheumatoid arthritis (1 case), malignant exophthalmos (1 case), Behçet's disease (1 case).

The mean duration of chemotherapy was  $35 \pm 22$  months (42 cases available - range 3-98 months), and the mean cumulative dose of chlorambucil was  $5.8 \pm 3.9$  g (35 cases available - range 0.3-18 g).

The mean interval from discontinuation of cytotoxic chemotherapy to leukaemia was  $38 \pm 28$  months (39 cases available - range 1-132 months). Various blood abnormalities were commonly encountered during the preleukaemic phase including pancytopenia, neutropenia, macrocytic anaemia, thrombocytopenia, monocytosis or mild myeloid leukaemoid reaction. Most of the 12 patients for whom bone marrow pictures were available exhibit increased cellularity with marked dysmyelopoiesis. Cytologic abnormalities involved the three lines, but erythroblastic changes were most striking. The cytological type of leukaemia is known in 43 cases. Most of these were of the myeloid types (37 cases). Response to antileukaemic therapy was poor. The mean duration of survival from onset of leukaemia was 2 months (24 cases available - range 0-8 months).

Finally regarding its leukaemogenic action, the prolonged use of chlorambucil for non-neoplastic diseases should be discouraged certainly restricted to severe, life-threatening forms.

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advanced Hodgkin's disease (IIIB and IVB). Skin reactivity was assessed by the combined use of purified protein derivative (PPD) and dinitrochlorobenzene (DNCB) skin tests. PPD and DNCB skin tests were chosen because PPD had been shown to be positive in 80% of the adult urban population in Turkey and 95% of normal population could be sensitized to DNCB [5]. PPD and DNCB skin testing procedures were carried out according to the methods previously described [5, 11]. The Ficoll-Hypaque density gradient method was used to separate lymphocytes and monocytes [2]. E-rosette-forming cells were enumerated by the method of Brada *et al.* [3]. Monocyte counts were calculated from percent monocytes

Table I. E-rosetting lymphocytes and monocytes in relation to anergy in Hodgkin's disease

Subjects		Mean counts/mm <sup>3</sup> ± SD	p
<i>E-rosetting cells</i>			
Patients	28	544 ± 449	< 0.001
Controls	19	1,173 ± 493	
Normergic patients	16	547 ± 332	> 0.05
Anergic patients	12	574 ± 701	
<i>Monocytes</i>			
Normergic patients	16	768 ± 485	> 0.05
Anergic patients	12	612 ± 497	

## Results and Discussion

Cutaneous anergy as defined by unresponsiveness to both PPD and DNCB was found in 43% of our patients. 13 of 14 (93%) of normal controls and 16 of 28 (57%) of Hodgkin's disease patients responded to either or both of these test materials ( $p < 0.01$ ). 2 of 7 patients with localized disease (stage IA, IIA) and 10 of 21 patients with advanced disease (stage IIIB, IVB) were found to be anergic. The difference was not significant ( $p > 0.05$ ,  $\chi^2 = 0.9$ ). We found no correlation between the clinical stage and skin anergy when 46 patients with Hodgkin's disease were skin tested previously [unpublished observations]. There was a significant decrease in the E-rosetting lymphocytes in our patients when compared with the normal controls ( $p < 0.001$ ) (table I) however no statistically significant difference in the mean number of E-rosetting lymphocytes could be established between the normergic and anergic patients. We did not find an increase in the mean number of blood monocytes in Hodgkin's disease cases. Furthermore, when normergic and anergic patients

were compared in regard to the blood monocyte counts, the mean number of monocytes was not higher in the anergic patients. We, therefore, conclude that the numbers of E-rosetting lymphocytes (T lymphocytes) or blood monocytes do not correlate with skin responsiveness in Hodgkin's disease.

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## Lack of Correlation between Skin Reactivity and T Lymphocytes and Monocytes in Hodgkin's Disease

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**Key Words.** Hodgkin's disease Monocytes Skin reactivity T Lymphocytes

**Abstract.** 28 untreated patients with Hodgkin's disease have been studied in respect to skin reactivity to purified protein derivative and dinitrochlorobenzene and blood monocytes and T Lymphocytes. Cutaneous anergy as defined by unresponsiveness to both purified protein derivative and dinitrochlorobenzene was found in 43% of patients and in 7% of the control group. Although there was a significant reduction in E-rosetting lymphocytes in Hodgkin's cases, we found no differences in the mean numbers of E-rosetting lymphocytes and monocytes between normergic and anergic patients. We, therefore, conclude that skin reactivity is not directly correlated with the total numbers of E-rosetting lymphocytes or monocytes in Hodgkin's disease.

The immunologic deficit in Hodgkin's disease involves mostly the cell mediated immunity. Depressed reactivity to skin test antigens *in vivo* [10, 14, 15], delayed homograft rejection [6, 9], inability to acquire contact sensitivity and accept the passive transfer of delayed hypersensitivity [1, 4, 11] and defective *in vitro* lymphocyte responses [7, 8] have been reported. Recently Schechter and Soehnlen [13] showed that the poor blastogenesis of lymphocytes in Hodgkin's disease was due to the presence of high concentrations of monocytes in the cell preparations. They suggested that the

inhibitory monocyte-lymphocyte interaction may be one of the causes of impaired cell-mediated immunity in this disease.

We have studied the correlation of skin reactivity and the numbers of E-rosetting lymphocytes (T lymphocytes) and monocytes in 28 untreated patients with Hodgkin's disease.

### Materials and Methods

28 patients with mean age of 32 years (range 8-70 years) and a median age of 28 years and 19 normal controls of corresponding ages were included in this study. 7 of the patients had localized disease (IA and IIA) and 21 patients had

<sup>1</sup> We wish to thank Dr. Hasan Yavuz for his help with the statistical analyses.

advanced Hodgkin's disease (IIIB and IVB). Skin reactivity was assessed by the combined use of purified protein derivative (PPD) and dinitrochlorobenzene (DNCB) skin tests. PPD and DNCB skin tests are chosen because PPD had been shown to be positive in 80% of the adult urban population in Turkey and 93% of normal population could be sensitized to DNCB [5]. PPD and DNCB skin testing procedures were carried out according to the methods previously described [5, 11]. The Ficoll-Hypaque density gradient method was used to separate lymphocytes and monocytes [7]. E-rosette-forming cells were enumerated by the method of Brube *et al* [3]. Monocyte counts were calculated from percent monocytes.

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## Lack of Correlation between Skin Reactivity and T Lymphocytes and Monocytes in Hodgkin's Disease

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<sup>1</sup> We wish to thank Dr. Hasan Yazıcı for his help with the statistical analyses.

## Effect of Thiamphenicol on Iron Absorption

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**Key Words.** Iron absorption · Ferrokinetics · Thiamphenicol

**Abstract.** The effect of thiamphenicol (a protein synthesis inhibitor) administration on iron absorption was studied in rabbits. Iron absorption was measured in a whole-body counter using  $^{59}\text{Fe}$ , and plasma iron turnover (PIT) was simultaneously determined with  $^{59}\text{Fe}$ . In comparison with the controls, the group receiving thiamphenicol showed no significant difference in PIT ( $p > 0.05$ ), while iron absorption was increased ( $p < 0.0001$ ).

### Introduction

After the introduction of the antibiotic thiamphenicol (methylsulfonyl analogue of chloramphenicol), it was recognized that this drug induced (reversible dose related) bone marrow depression during the treatment. This effect is probably related to protein synthesis inhibition at the mitochondrial level [2, 5]. In the present investigation, iron absorption was determined using an *in vivo* method. Simultaneously plasma iron turnover (PIT) was also measured after thiamphenicol administration.

### Materials and Methods

#### Animals

The animals used were 4-kg male Albino rabbits aged 4-5 months (Lamotte SA, Aarau, Switzerland).

#### Experimental Procedure

A group of 6 rabbits received 500 mg/day thiamphenicol intramuscularly (Urfamkyn® Imphenizam SA, Lamotte Lognon, Switzerland) for 3 days and then 250 mg/day for 3 days. The ferrokinetics were performed 5 days after beginning the administration of thiamphenicol. This drug did not produce any alteration in the weight or consistency of the feces. These rabbits and the controls (10 animals) were fed on normal diet for rabbits (Lacta SA, Coppet, Switzerland), without antibiotics. Food and water were supplied *ad libitum*.

#### Ferrokinetics and Iron Absorption Studies

Iron absorption and PIT were measured on the same day as previously described [1]. PIT was determined with  $^{59}\text{Fe}$  and expressed as nmol/kg/day. Iron absorption was measured in whole-body counter for animals using  $^{59}\text{Fe}$ .

#### Hematological and Serological Determinations

Hematological determinations were done with laser ray counter (HemaC 830 L, Ortho Diagnostics).



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changed. Figure 1 shows the results of PIT and iron absorption. The PIT in the group treated with thiamphenicol was not changed in comparison to the control group ( $p > 0.05$ ). Nevertheless, the iron absorption increased significantly ( $p < 0.0001$ ) in comparison to the controls.

### Discussion

After the administration of thiamphenicol, according to the dose proposed by Nijhof *et al.* [3] we observed a decrease in reticulocytes, suggesting a depression of erythropoiesis. The plasma iron was increased and the plasma iron turnover remained unmodified in comparison to the control group. The diminution of erythropoiesis induced by thiamphenicol is probably related to a toxic effect of this drug on protein synthesis at the mitochondrial level: it inhibits respiration, ATP generation, DNA synthesis and cytochrome-C-oxidase activity in marrow cells [2, 5]. Treatment with thiamphenicol also increases iron absorption, despite normal PIT. These data suggest that thiamphenicol can alter the control of iron absorption at the level of the intestinal mucosa. We have not investigated the exact mechanism of this alteration, but it is possible that this drug interferes with normal protein synthesis at this level.

In fact, the level of protein synthesis in the mucosal cell can play an important role in the control of iron absorption. Iron transfer from the intestinal lumen to the portal blood stream involves two phases that can be separated from one another. The initial passage of iron across the brush border into the mucosal cell occurs by passive diffusion. The subsequent transfer of some of this iron

blood is inversely correlated with the quantity of ferritin in the mucosal cell and probably directly correlated with an iron carrier identified by some authors as transferrin-like protein [4]. It is conceivable that if protein synthesis decreases due to thiamphenicol, the iron that crosses the membranes of the intestinal mucosal cells is not retained by the intracellular ferritin and can cross the cells to the capillar circulation.

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tics Instruments, N J). The serum iron concentration was measured according to the recommendations of the International Committee for Standardization in Hematology Reticulocytes were counted in 2,000 cells stained with methylene blue and counterstained with Wright's stain.

#### Statistical Evaluation

Statistical analysis was performed with nonparametric tests: the Wilcoxon test for paired differences and the Mann-Whitney ranking for unpaired values.

## Results

Table I shows the results of the hematological determinations in the group treated with thiamphenicol before and after administration of this drug and in the control group. Reticulocytes were decreased after the antibiotic treatment in comparison to the values before administration or compared to the control group. Serum iron increased and hemoglobin remained in-

Table I. Hematological determinations

	Thiamphenicol group		p <sup>1</sup>	Control group (n = 10)	p <sup>2</sup>
	before treatment (n = 6)	after treatment (n = 6)			
Hemoglobin g/dl	12.0 ± 1.5	10.7 ± 0.5	n.s.	12.1 ± 2.1	n.s.
Hematocrit %	38.0 ± 2.1	29.8 ± 1.1	< 0.001	38.7 ± 3.2	< 0.01
Reticulocyte count, %	3.9 ± 1.2	0.8 ± 0.2	< 0.001	3.7 ± 1.7	< 0.01
Serum iron, $\mu$ mol/l	22.1 ± 5.2	48.7 ± 8.7	< 0.001	20.8 ± 6.1	< 0.01

n.s. = Not significant ( $p \leq 0.05$ ) n = number of rabbits.

p values were obtained using the Wilcoxon test between data before and after treatment with thiamphenicol.

p values were obtained using the Mann-Whitney test between data after thiamphenicol treatment and control group.

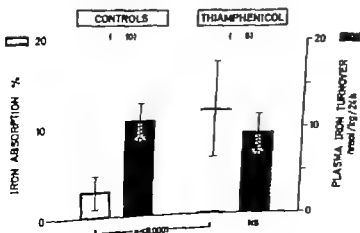


Fig. 1 Effect of thiamphenicol administration on iron absorption and PIT. Open bars = mean iron absorption  $\pm$  1 SD. Hatched bars, mean plasma iron turnover  $\pm$  1 SD. In parentheses, the number of animals.

Table 1. Laboratory and clinical findings in 7 subjects with HO belonging to one family (pedigree in fig. 1)

	Father	Brother case 1	Brother case 2	Sister	Aunt case 3	Cousin	Grandmother
Hb, g/l	130	133	142	140	136	143	133
Reticulocytes, %	1.3	1.8	0.7	2.2	4.0	1.5	5.0
S-bilirubin, $\mu\text{mol/l}$	10	10	9	10	11	14	13
Ery-ovalocytes, %	58	30	40	28	50	70	25
Ery-microspherocytes, %	0	3.5	10.0	0.5	0	0.5	0
S-haptoglobin, g/l	0	0.01	0.02	0.01	0	0.50	0
Osmotic fragility of erythrocytes	normal	'tail'	'tail'	normal	n.t.	n.t.	n.t.
Autohaemolysis							
Without glucose, %	9.0	7.2	7.0	2.6	n.t.	n.t.	n.t.
With glucose, %	1.9	1.3	1.7	0.7	t.	n.t.	t.
G-6-P-D activity	normal	normal	normal	normal	n.t.	n.t.	n.t.
Symptoms and signs	none	splenic rupture	splenic rupture	spleno- megaly	cholelithias- is and previous anaemia	none	cholelithiasis

G-6-P-D = Glucose-6-phosphate-dehydrogenase in erythrocytes 'tail' = increased fragility in the range 0.5–0.6% NaCl t. = not tested.

Mean value of three senior medical technicians analyses. 400 erythrocytes were counted (variation of the mean  $\pm 5\%$ ).

that the family probably belonged to the genealogical type where the defective gene is coupled to the theta locus [2].

#### Comment

An exact grouping of our subjects with HO according to *de Gruochy et al.* [5] is not possible since splenectomy had been performed on 2 of them. They probably belong to group 1 although group 2 cannot be excluded since the father's autohaemolysis test was slightly increased.

We have found no previous report of ruptured spleen as presenting symptom of HO. We believe that a fragile spleen, due to HO contributed to the ruptures in the 2 brothers. We have discussed a prophylactic splenectomy in the sister who has a moderately enlarged spleen, but have since decided against the idea.

As homozygosity gives severe haemolytic anaemia with haemolytic crises already during the neonatal period [7] we have given the family some basic genetic information.

## Hereditary Ovalocytosis and Splenic Rupture

Bengt Kjellman Christer Larsson and Elisabeth Tibblin

Departments of Paediatrics and Clinical Chemistry Unit of Haematology Kärnsjukhuset, Skövde

**Key Words.** Hereditary ovalocytosis Splenic rupture

**Abstract.** A family with hereditary ovalocytosis (HO) is described. The probands, 2 brothers, had splenic rupture after modest trauma as presenting symptoms. 7 members of the family had HO. The sister of the probands had a moderately enlarged spleen. The other members proved normal on routine clinical examination.

This communication is based on a study of a family with hereditary ovalocytosis (HO). The probands, 2 brothers, had splenic rupture after comparatively mild trauma. In this study an ovalocyte is defined as an erythrocyte with an axial ratio of less than 0.78 [7] and ovalocytosis as a condition with ovalocytes in excess of 25% of the total number of erythrocytes [6]. Osmotic fragility was estimated according to Dacie [3] *in vitro* tests of autohaemolysis according to Dacie and Selwyn [4] and the activity of glucose-6-phosphate dehydrogenase as described by Berger [1].

**Proband 1** a boy was admitted to the hospital at the age of 10 years because of streptococcal tonsillitis. His admission coincided with that of his brother (proband 2). 1 year earlier he had been splenectomised because of splenic rupture after a slight trauma during a ball game. On routine blood examination, we found a pronounced ovalocytosis.

**Proband 2** a boy was admitted at the age of 15 years after a traffic accident with splenic rupture being his only injury. Due to our knowledge of his brother we investigated him with respect to ovalocytosis.

An investigation of the family disclosed the clinical and blood morphological findings presented in figure 1. Results of laboratory studies are given in table I. An analysis of the linkage between the rhesus blood group and HO showed

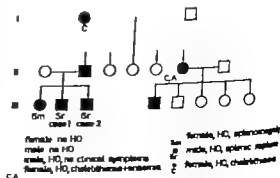


Fig. 1. Pedigree. Three generations (I, II and III) of family with HO

## Acquired Pelger Huët Anomaly Limited to Eosinophils

Sir,

We wish to report a new case of acquired Pelger-Huët anomaly confined to eosinophils.

This unusual alteration was observed in a 68-year-old woman with typical agnogenic myeloid metaplasia. The laboratory data revealed moderate normocytic normochromic anemia, thrombocytopenia and leukocytosis ( $11.10^9/l$ ). Bone marrow sections demonstrated moderately severe myelofibrosis. The leukocyte alkaline phosphatase score and the chromosomes were normal. Peripheral blood smears showed erythrocytes with striking tear-drop poikilocytosis, a leukoerythroblastic reaction and many giant platelets and megakaryocytic fragments. Eosinophilic cells were 2%, all of them pre-

senting round pyknotic nuclei with clumped chromatin (fig. 1) while neutrophils had a normal nuclear segmentation. This phenomenon was never noted during previous admissions. Moreover the patient had never been treated with myelotoxic agents.

Acquired or pseudo Pelger-Huët anomaly limited to eosinophilia, first described by Kay *et al* [2] in 2 cases of myeloproliferative syndrome, was recently reported by us [1] in a patient with acute myeloblastic leukemia. Because of this further observation we believe that the anomaly is not so rare as previously appreciated, and it is probably an intrinsic part of various conditions of disordered myelopoiesis.

Sincerely yours,

V Possalanza and F Tosato

### References

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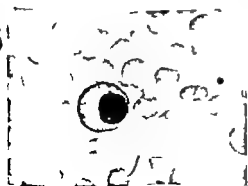


Fig. 1. A pseudo Pelger-Huët eosinophil. Peripheral blood. May-Grunwald-Giemsa stain

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Fig. 1. A pseudo Pelger Huët eosinophil. Peripheral blood May-Grunwald-Giemsa stain.



## Book Reviews

*N Selwood and A Hedges*  
**Transplantation Antigens**  
**A Study in Serological Data Analysis**  
Wiley Chichester 1978  
VIII + 147 p £9.50  
ISBN 0-471-99657-2

The urgent need which arose to clarify the antigenic system of leucocytes has led to the collection of many data. This book is an attempt to simplify the description of methods which have been used to extract information from basic serological data. On more than 140 pages the authors describe the history and development of HLA-serology the search for facts, methods of analysis, the development of a numerical approach to the specific problems of HLA-analysis and its application and speculate in the final chapter on the source of heterogeneity in serological results. Although the book is directed generally to serologists, anthropologists and geneticists may also be interested because the principles outlined are not exclusively related to experts in HLA-serology. The book is clearly written and will be very valuable for those workers experienced in the world of immunohaematology and mathematics.

S. Seidl, Frankfurt am Main

*R. N Barnett*  
**Clinical Laboratory Statistics** 2nd ed  
Little, Brown, Boston 1979  
XII + 237 pp. US \$ 17.50  
ISBN 0-316-08196-5

The 23 chapters of the text are divided into five parts. Principles of Statistical Analysis, Application of Statistics in the Clinical Laboratory, Applications of Statistics in Patient Care, Statistics for Manufacturers, and Statistics in Medical Literature. Appendix tables provide adequate information for interpreting F tests, t tests, 2 by 2 contingency tables for small series of values, and for computing confidence limits for standard deviation. The 2nd edition has been extended to include much new material (partially contributed by J M

Weisbrof) linear regression, normal values with reference to Gram-Charlier Analysis, Poisson distribution, quality control in hematology and other topics. All the specific applications are described by means of familiar laboratory examples. The authors succeed in bringing together both the medical laboratory and the statistical viewpoints. In this sense the book is recommended to the many persons who are intimately concerned with clinical laboratory problems and who require an understanding of statistics to help them cope with those problems.

M. Dozzi, Bern

*G Marké M Seligman and M Tablani*  
**Recent Results in Cancer Research**  
Vol. 64 Classification, Categorization,  
Natural History  
Springer Berlin 1978  
370 pp 168 fig., 88 tab. DM 88.  
ISBN 3-540-08330-X

'Lymphoid Neoplasias' was the subject of a CNRS International Colloquium which was held in Paris in June, 1977. Papers presented at the meeting have been published in volumes 64 and 65 (see below) of the renowned RRCR series. In this first part, contributions by an international group of specialists address themselves to the controversial problems of nomenclature and classification as well as to a series of interesting animal and clinical models of lymphoid neoplasia in a broader sense. A dozen each of presentations deal with morphological and/or immunological/physicochemical criteria, respectively which are currently used in attempts at classifying non-Hodgkin lymphomatous states: fortunately the chairman of the first session contributed a 'conversion table' to facilitate comparisons between the five now widely used classifications (Rapaport, Loken-Collins, Kiel, British Lymphoma Study Group and WHO). This volume offers an adequate picture of the state of affairs in 1977. It is of interest to note that overall there is more accord than discord in the definition of important disease entities within the group of lymphoid neoplasias.

M. Hew, Bern

*G. Maht, M. Seligman and M. Tscham*  
Recent Results in Cancer Research  
Vol. 65: Clinical and Therapeutic Aspects  
Springer Berlin 1978  
260 pp., 110 tab., 72 fig., DM 60.-  
ISBN 3-540-08831-8

This companion volume to 'Lymphoid Neoplasias I' characteristically is less voluminous by 200 pages, is the first part, results of clinical and histological staging procedures in large series of juvenile and adult forms of non-Hodgkin lymphomas are presented. The relative value of staging appears to become appreciated after 10-year period of experience with cases in adults, while extensive surgery in children proved rarely of value. As evident from the second part which deals with therapeutic aspects of the disease groups, clinical trial results are difficult to compare because of differing terminologies and differing definitions of response, duration and survival. It is urged that reporting of treatment results be standardized. For the time being therapeutic attempts do not reflect advanced histological and functional classifications but are based almost world-wide on the oldest and relatively simple scheme proposed originally by Rappaport. Combined efforts of European and American investigators should lead to an international consensus with eventual benefit for the patients.

Both volumes are of interest to pathologists, clinicians and oncologists. M. Hess, Bern

The volume provides an excellent review of the present day knowledge and is most valuable source of information for laboratory investigators and clinical hematologists interested in this field of research. H. R. Marti, Aarau

*E. J. Freireich et al. (eds.)*  
Leukemia and Lymphoma  
Grune & Stratton, New York 1978  
X + 358 pp. US\$ 24.50  
ISBN 0-8089-1166-X

This collection of 18 symposium articles in book form is divided into two sections. The first deals with immunologic aspects which range from experimental models through immunodeficiency and pathogenesis, immune aspects in viral-induced leukemias and lymphomas, cellular types and clones in regard to immunologic criteria, tumor associated antigens and their reactivity to immunotherapy of leukemia and lymphoma. The second section is somewhat more clinically oriented with the heading of 'Treatment, Diagnosis and Biology'. Here the approaches to treatment of the lymphomas, acute leukemia and chronic granulocytic leukemia and their results are presented. Also, chapters on the immunology of bone marrow transplants, chromosomal and cell culture studies, cell surface regulation of hematopoiesis, the viral etiology of this disease group and drug resistance in experimental therapeutic and genetic models are to be found.

The authors are all very competent specialists in highly developed fields of research. Complete digestion of all the material presented is not always possible for the uninitiated without resort to more basic literature. However with their ability to clearly present experimental data, theories and opinions, the authors provide the reader with an understanding of the achievements, problems, current limitations as well as future possibilities in these areas. Present concepts as to the etiology pathophysiology and treatment of this disease group are well covered within the 357 pages. The abundance of unanswered questions also becomes very apparent. The basis for further studies and the direction of such endeavors are indicated.

This volume will certainly be of interest to hematologists and oncologists. R. Sonntag, Bern

*G. Stamatoyannopoulos and A. W. Nienhuis*  
(eds.)  
Cellular and Molecular Regulation  
of Hemoglobin Switching  
Grune & Stratton, New York, 1979  
792 pp. US\$ 66.50  
ISBN 0-8089-1159-7

The book contains the proceedings of conference held June 19-21, 1978, in Seattle, Wash. U.S.A. The 51 contributions given by outstanding experts are divided in 3 parts entitled 'Developmental hemoglobins in man and animal models', 'Hemoglobin switching and erythroid cell differentiation' and 'Molecular biology of hemoglobin switching'.

H von Voss

**Prüfleistung und Anwendung von Thrombozytenfunktionswerten in der Kinderheilkunde**  
Forschungsberichte des Landes  
Nordrhein Westfalen,  
Heft 2788/Fachgruppe Medizin  
Weindeutscher Verlag, Opladen 1979  
VI - 174 pp 34 fig., 41 tab DM 29.-

H. von Voss, an experienced pediatric hematologist at Düsseldorf University discusses special laboratory problems dealing with platelet function defects. He thoroughly explains in a very lucid style the criteria used in establishing the reliability of conventional platelet function tests (bleeding time, clot retraction, spreading, aggregation, retention tests and adhesiveness) as well as the new test methods now in use (e.g. malonyldialdehyde production of platelets). When examining newborns and infants for hemostatic disorders, the number of tests that can be performed is obviously limited by the amount of blood obtainable for such studies. However the combination of reliable tests is vital since acquired and inherited disturbances of platelet function are often found to be the cause of severe bleeding complications. Even drug-induced bleeding complications can be expected in children (e.g. aspirin for rheumatic illnesses, ergenyl for epileptic seizures), as demonstrated in detail by the author.

To support his arguments, the author mentions the results of his own *in vitro* assays and indicates their normal range. In spite of numerous detailed data, the book, by and large, presents the various problems in a very clear and succinct manner enabling the clinician to judge the methods and easily find answers to specific questions. The book can be recommended to all physicians performing laboratory research, especially in pediatric hematology. Scientists working in the field of platelet abnormalities may find the book interesting because of its excellent review of the current literature.

E. Wenzel, Homburg

S. L. Lamberg and R. Rothstein (eds.)  
**Hematology and Urinalysis.**  
Functional Medical Laboratory Technology  
AVI, Westport 1978  
VIII + 174 pp. US \$ 11.-  
ISBN 0-87055-268-6

This 174-page laboratory manual introduces students in medical technology to commonly performed basic hematology tests and analyses. The manual is unique in that it describes and depicts step by step the performance of the most important simple tests. 25 pages alone are devoted to the detailed description of the methodologies of blood collection by venipuncture and by fingertip puncture. The numerous illustrations are instructive and useful, procedures are simply and well described. For each procedure there is an extensive discussion of the theoretical aspects and of possible errors. Each chapter is followed by a section of programmed questions. The concept of such a manual is an excellent one and the reader realizes at once that the authors are experienced teachers of medical technology students.

Unfortunately the authors have not taken the precaution to have their manuscript reviewed by a competent hematologist and biochemist. The manual abounds with obsolete dogmas, inaccurate expressions and outright wrong statements. Some of the obsolete methods described, such as the fingertip puncture bleeding time should be replaced by currently used tests (Ivy or Template bleeding time). Since the authors discuss modern mean corpuscular values it is redundant to waste 3 pages on obsolete erythrocyte indexes and beyond that to illustrate erythrocytes with a colour index of 0.1 (wrt). Before I could ever recommend this manual to students, dozens of wrong statements would have to be corrected in a second edition. I mention just a few examples to aid to be a cheating agent (p. 29): eosin, an acid stain is said to dissociate too much at a more acidic pH (the contrary is correct, p. 33) we are told that the number of lobes of the polynuclear neutrophil varies with the age of the cell (p. 39) that an increased viscosity of the plasma decreases erythrocyte sedimentation rate (compare that to multiple myeloma where it is high and DIC where it is zero, p. 98) states with increased serum albumin leading to diminished sedimentation rate do not exist in human pathology (p. 107): it is extremely poor technique to use the Rees-Ecker platelet count with a multiplicative factor of  $10^4$  what about the patients with thrombocytopenia? (p. 108): It is incorrect that platelets are included in the red cell count when using Coulter counters, but that this does not matter (thrombocythemia? p. 128) hyperchromic red cells do or exist (p. 140) myoglobin is not a hemo-

globin derivative (p.144); overweighed (lighter than water) toluene is said to increase specific gravity of urine; normal specific gravity (of urine) does not equal isothermia. In addition, awkward statements such as: 'urine is the most significant way of elimination of substances of cellular metabolism produced in excess, such as water and electrolytes' and 'the increase in specific gravity is not the same for every kind of solute, as some solutes are ionized and others are not' would have to be properly formulated. The example given on quality control concerning the haemoglobin determination is outrageous. The standard globontrol is said to contain 15 g/100 ml, with a standard deviation of 5 g/100 ml (!). The authors appear to be content if the daily checks of the globontrol standard fall within 13-18 g/100 ml and state 'values are well within normal control limits as they cluster around the mean'. When the globontrol standard of 15 g/100 ml yields a value of 7 g/100 ml in the authors' example, they state 'values are in normal range but fluctuate'. I would fire medical technologists who produce such quality control charts about immediately alarming his superiors.

F. Sackmann

B. Blomhök and L. A. Henson (eds.)

Plasma Proteins

Wiley Chichester 1979

XVI+401 pp., US \$ 22.50

ISBN 0-671-99730-7

This volume contains contributions on the structure and function of plasma proteins by 23

authors. The book is an updated translation of the original 1976 Swedish edition by AB Kahl. It is evidently a Swedish book on plasma proteins reflecting particularly the outstanding contributions of Swedish investigators in this field. The review on the history of plasma protein chemistry is followed by a description of the Kahl (and other) industrial plasma fractionation methods. The chapter on transport proteins provides information on albumin, lipoproteins, ceruloplasmin, transferrin, haptoglobin, hemopexin, transcobalamin, retinol-binding protein, transthyretin and proteins binding thyroid hormones. An extensive section on immunoglobulins includes also immunological diseases, transplantation and blood group serology. Of the coagulation factors, fibrinogen and thrombin received the most attention. In addition, survey is given of the methods used for assay and characterization of plasma proteins as well as of their diagnostic use.

The content and the form of the individual chapters are quite unbalanced. The chapters on immunoglobulins, methods and clinical applications are very readable texts for the student, whereas others, especially descriptions of transport proteins and coagulation factors, are rather comprehensive reviews of recent work (up to 1977) for the expert in the respective fields. Unfortunately the references, added at the end of the book, are sometimes quite confusing and inappropriate. For example, 6 references mentioned in the chapter on AHF are missing in the reference list while, of those listed, only 4 out of 18 are referred to in the text.

M. Fariza, Bernes

H von Voss

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### *In vitro* Autohaemolysis during Pregnancy

D O Ho-Yen, D M Clark, Margaret A R Thomson, R A Brown and H B Goodall

University of Dundee and Ninewells Hospital, Dundee

**Key Words.** Anaemia, haemolytic Autohaemolysis Erythrocytes Pregnancy

**Abstract.** *In vitro* autohaemolysis with and without the addition of glucose was performed on normal pregnant women chosen at random and on non-pregnant women of comparable age. Autohaemolysis was significantly greater in the pregnant than the non-pregnant. The relationship between the stage of gestation and *in vitro* autohaemolysis in normal pregnancy may be fitted by a quadratic curve.

#### Introduction

When normal erythrocytes are incubated under sterile conditions, there is only slight haemolysis after 48 h [1]. If glucose is added to the blood prior to incubation, the amount of haemolysis is reduced. The degree of *in vitro* autohaemolysis is increased by abnormalities of the red cell membrane or of energy production. Thus the autohaemolysis test has been extensively used in the preliminary investigation of haemolytic anaemias. Recently it has been suggested that the availability of spot tests for specific enzyme deficiencies has made the autohaemolysis test obsolete [2]. Enzymic assays have certainly limited the use of the autohaemolysis test in laboratories where these assays are available; however there is still a place for the autohaemolysis test in detecting

membrane abnormalities and enzyme deficiencies for which there are no screening tests. We have recently investigated two such cases [3, 4]. In investigating these cases and another with spherocytic haemolytic anaemia during pregnancy we found variations in the amount of autohaemolysis in normal pregnancy women who were used as controls. The purpose of this paper is to report the results of the autohaemolysis test in non-anaemic pregnant women, as there is practically no information available on the subject.

#### Materials and Methods

The autohaemolysis tests were performed in standard way with and without glucose [5]. 20 normal non-pregnant women and 40 normal pregnant women were tested. The pregnant women

## Announcement

Lady Davis Institute for Medical Research -  
Jewish General Hospital  
Symposium on Iron Overload -

### Basic Mechanisms and Treatment

To be held in Montreal on August 16, 1980, as a satellite symposium to the International Society of Hematology Congress.

Speakers will include: *P. Allen* (New York), *A. Cerami* (New York), *R. Grady* (New York),

*C. Hershko* (Jerusalem), *A. Jacobs* (Cardiff), *H. Munro* (Cambridge), *A. Neukirch* (Bethesda), *P. Ponka* (Montreal), *G. Richter* (Rochester), and *H. Schulman* (Montreal).

Attendance at the Symposium will be free of charge, but advance registration will be required. Deadline for registration, July 1 1980. For program and registration contact: Dr. *H. Al. Schulman*, Lady Davis Institute for Medical Research, Jewish General Hospital, 3755 Avenue Cote St. Catherine Road, Montreal, Que. H3T 1E2 (Canada).

is consistent with dilution of cell contents accompanying spherocytic swelling. It has also been suggested that a fall in plasma colloid osmotic pressure may cause the sphering [9].

The changes in autohaemolysis during pregnancy (fig. 1) are of timing similar to changes in MCV, osmotic fragility and intracellular sodium. The sphering probably results in increased lysis during incubation *in vitro*. This lysis is reduced by addition of glucose, but in pregnant women remains greater than in the non-pregnant. The peak of these erythrocytic changes near term has not so far been adequately explained, but some of the changes in the erythrocytes near term may be related to the age of the red cell population, for Pritchard and Adams [12] found, at that phase of gestation, small increases in reticulocytes and in erythrocytic glycolysis and cholinesterase activity.

The autohaemolysis test is reliable only if carried out with strict attention to detail, particularly to the elimination of contamination [4], and it is also, as with other tests, important that each laboratory establish its own normal range. The precision of the method is enhanced if carried out by the same individual [1]. The results of this paper emphasise the pattern of autohaemolysis rather than the absolute values.

The interpretation of increased autohaemolysis is difficult. A good correlation between autohaemolysis *in vitro* and red cell destruction *in vivo* has been found in congenital and acquired haemolytic anaemias [11], but red cell survival in the latter half of normal pregnancy appears to be normal [12] so the increased *in vitro* autohaemolysis in apparently normal pregnant women beyond non-pregnant levels need not necessarily imply underlying haemolytic disease. Nevertheless, the autohaemolysis test may

still be of use in the assessment of patients with unexplained haemolytic anaemia in pregnancy when the use of radioisotopes are better avoided, provided that the effect of glucose is tested [4] and that the results are interpreted in relation to the pattern of autohaemolysis now shown.

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were chosen at random at different gestation periods. Each was tested once. None were anaemic (haemoglobin levels above 12 g/dl)

## Results

Without additives, the range of *in vitro* autohaemolysis in the non-pregnant women was 0-1.8% (mean = 1.0 and SD = 0.5) and in the pregnant women the range was 0-4.0% (mean = 2.0 and SD = 1.0). With the addition of glucose, the range of *in vitro* autohaemolysis in the non-pregnant controls was 0-1.0% (mean 0.5 and SD = 0.3) and in the pregnant controls the range was 0-1.7% (mean = 1.0 and SD = 0.5). When both pairs of results were analysed by the Student's *t* test, it was found that there was a very significant difference between pregnant and non pregnant patients, with and without glucose ( $p < 0.01\%$ ).

When the percentage autohaemolysis without any additives is plotted against the duration of gestation, a definite pattern

Table I. Analysis of variance on the results of autohaemolysis without additives in pregnant women at various stages of gestation

Source of variability	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Due to fitted curve	23.33	2	12.61	16.81
Lack of fit	4.68	15	0.32	< 1
Pure error	16.55	22	0.75	
Total	46.56	32	-	

Significant at 1% level.

emerges (fig 1). A quadratic curve can be fitted to the data and an analysis of variance carried out (table I). Thus, the relationship between percentage autohaemolysis and stage of pregnancy as shown by the fitted curve, is significant at the 1% level. There was a similar trend with glucose added, but this is not statistically significant.

## Discussion

Various changes occur in the erythrocytes during apparently normal pregnancy. Thus, Dieckmann and Wegner [6] found a gradual increase in mean corpuscular volume (MCV) until the 35th week, with a fall thereafter to normal. Although their work was done before modern accurate haematological analyses were developed, it is broadly supported by Chanarin *et al.* [7] using the Coulter Counter Model S. An increase in osmotic fragility possibly related to slight spherocytosis, takes place about the same stage of gestation [8, 9]. A fall in intra-erythrocytic potassium until the 32nd week [10]

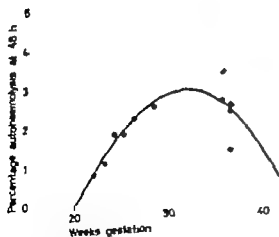


Fig. 1. Percentage autohaemolysis plotted against weeks of gestation (fitted curve,  $y = -20.2 + 1.47x - 0.023x^2$ ).

## Hemoglobin Hasharon [ $\alpha_1 47$ (CD5) Asp $\rightarrow$ His $\beta_1$ ] Linked to $\alpha$ Thalassemia in Northern Italian Carriers

### Hematological and Biosynthetic Studies<sup>1</sup>

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**Key Words.**  $\alpha$ -Chain genes  $\alpha$ -Thalassemia  $\beta$ -Thalassemia Hb Hasharon

**Abstract.** This report is concerned with the evaluation of hematological parameters and of both relative (%) and absolute (mean pg/cell) quantities of the abnormal Hemoglobin (Hb) Hasharon in 53 heterozygous carriers and 7 double heterozygotes for Hb Hasharon and  $\beta$ -thalassemia from 43 apparently unrelated families living in the province of Rovigo (northern Italy). Biosynthetic studies are also reported. The data strongly suggest the presence of an  $\alpha$ -thalassemia-2 determinant closely linked to the  $\alpha$ -Hasharon-chain locus. Selective advantage of heterozygotes carrying such  $\alpha$ -haplotype would explain the relatively high frequency of Hb Hasharon (0.23%) in northeastern Italy, a past-endemic malaria region. The interaction between Hb Hasharon and  $\beta$ -thalassemia results in preferential decrease of the abnormal Hb level.

### Introduction

The genetics of the human hemoglobin (Hb)  $\alpha$ -chain has been of great and continuing interest since the first suggestion, provided by Lehmann and Carrell [11], that the duplication of the  $\alpha$ -chain structural genes is an almost general feature in humans. Direct support for this hypothesis came recently by the identification of two tightly linked  $\alpha$ -chain loci in human cellular DNA by restriction endonuclease mapping [16]. Since

the normal  $\alpha$ -chain gene products are structurally indistinguishable, both as globin chains and as mRNA molecules [7] the duplication is thought to have been a quite recent event in terms of molecular evolution, inasmuch as other globin chains which most probably have as well originated by gene duplication (the  $\beta$ -  $\gamma$ -  $\delta$ - and possibly the embryonic chains) have accumulated differences in their amino acid sequences. At present, there appears to be no well-documented evidence of populations having only one  $\alpha$ -chain gene per haploid genome without concomitant  $\alpha$ -thalassemia [22], and it remains therefore unclear if the  $\alpha$ -gene dupli-

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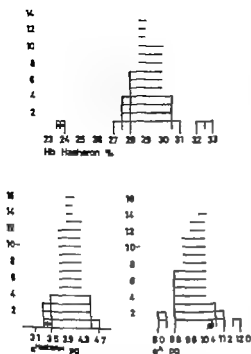


Fig. 1. Histogram representation of the Hb Hasharon percentages and of the absolute amounts of  $\alpha$ - and  $\beta$ -chains in the 53 heterozygous carriers. An asterisk indicates in the three histograms the single 23% Hb Hasharon carrier.

ces (RBC, MCV, MCH) were in general consistent with mild microcytosis, very mild erythrocytosis, and low mean cell Hb content. Slight anisocytosis was occasionally observed, whereas osmotic fragility was rarely slightly decreased, and there was no evidence of inclusion bodies in red cells on supravital staining. A complete table with hematological and hemoglobin data of the carriers is available on request.

In order to determine the significance of the alterations of RBC indices, the hematological data were compared to those of a group of normal subjects of comparable number, age and sex distribution by a computer-estimated one-way analysis of variance (table I). The distribution of RBC indices of both classes is shown in figure 2. The statistical analysis indicated that Hb Hasharon carriers have alterations of RBC indices towards microcytosis and low mean cell Hb content. The red cell count is slightly but significantly increased and, therefore, the total Hb value does not significantly differ from that of the normal control sample. These findings, together with normal Hb A<sub>2</sub> and serum iron levels strongly suggest the presence in these subjects of a concomitant

Table I. Comparison (one-way analysis of variance) between the variabilities of the RBC indices of the Hb Hasharon carriers and of the normal controls.

	RBC ( $10^{12}/\text{liter}$ )	Hb, g/dl	MCV fl	MCH, pg
Hb Hasharon heterozygotes (n=53)	$5.127 \pm 0.063$	$14.14 \pm 0.018$	$77.99 \pm 0.54$	$27.53 \pm 0.22$
Normal controls (n=53)	$4.746 \pm 0.063$	$14.13 \pm 0.17$	$90.09 \pm 0.60$	$30.07 \pm 0.22$
Analysis of variance				
F value	22.66	0.01	224.52	38.65
p value	$10^{-4}$	1	$<10^{-4}$	$<10^{-4}$

RBC = Red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin. Mean  $\pm$  SE values.

Bartlett's test for homogeneous variances was always not significant ( $p > 0.05$ ).

cation is already generally fixed in humans or if a normal (i.e. non-thalassemic) single  $\alpha$ -gene haplotype does actually exist, at least in certain populations. Thus, data concerning informative families with  $\alpha$ -chain structural Hb variants are of continuing interest.

Hb Hasharon [ $\alpha_2\gamma_2$  (CD5) Asp $\rightarrow$ His] $\beta_2$ ] is an electrophoretically slow moving Hb variant causing no clinical alteration in heterozygotes. Since its discovery in a Hebrew family of Ashkenazy origin [8] it was found in a number of carriers belonging to two main ethnical groups: (i) eastern European or Ashkenazy Jewries in whom the amount of Hb Hasharon ranged from 14 to 22% of the total Hb [reported by Lehmann and Vella 12] (ii) north Italians, in whom Hb Hasharon accounted for 30–40% of the total Hb [1] a level significantly higher than that found in Ashkenazy carriers.

The possibility that Hb Hasharon mutation occurs in Italian carriers on an  $\alpha$ -haplotype having a single active  $\alpha$ -chain locus is the subject of the present report.

## Materials and Methods

### Patients

53 Hb Hasharon carriers and 7 double heterozygotes for Hb Hasharon and  $\beta$ -thalassaemia from 43 apparently unrelated families of northern Italian origin (Polesine, the region of the Po river delta) have been studied. 8 out of these 43 families had already been identified in a previous report [1]. The abnormal Hb carriers were identified by routine cellulose acetate electrophoresis of hemolysates during  $\beta$ -thalassaemia screening programs carried out on schoolchildren living in the province of Rovigo (Polesine), followed by family studies.

### Hematological and Structural Studies

Venous blood was collected in EDTA, and standard hematological methods, including determination of the RBC indices by a ToA Microcell

counter CC-108 (ToA Medical Electronics Ltd, Japan), were employed [4]. Red cell hemolysates were examined by electrophoresis on cellulose acetate in a Tris-EDTA-borate buffer pH 8.4, and the relative amounts of Hb Hasharon and  $A_2$  were estimated by elution [13]. Determinations were done in triplicate with a mean coefficient of variation of less than 3%. The level of Hb F was estimated by alkali denaturation [18]. Globin was obtained from whole hemolysates by acid acetone precipitation, and examined by cellulose acetate electrophoresis in 0.025 M Na-barbiturate buffer pH 8.0 containing 6 M urea. Isolation of the abnormal  $\alpha$ -chain, identification of the abnormal tryptic peptides by paper fingerprinting, and amino acid analysis followed procedures described elsewhere [14].

### Globin Synthesis

Biosynthesis studies were carried out on 17 Hb Hasharon carriers from 9 families. 1.0 ml of packed red cells washed in reticulocyte saline were incubated for 60 min in a medium supporting protein synthesis and containing 100  $\mu$ Ci of  $^3$ H-leucine (Amersham, UK, spec. act. > 100 Ci/mmol) [9]. Whole-cell globin was prepared by acid acetone precipitation and chromatographed on CM-cellulose (Whatman CM 32) according to Clegg et al. [2]. The radioactivity of each fraction was assayed by liquid scintillation [9].

## Results

### Hematological and Hemoglobin Studies

Heterozygous carriers for Hb Hasharon had no relevant clinical symptoms. The percentage of Hb Hasharon ranged from 27.4% to 32.37% of the total Hb (mean  $\pm$  SE,  $29.02 \pm 0.16\%$ ) with a single value of 23.75%. The distribution of the percentage values, which is clearly unimodal, is shown in figure 1. The Hb  $A_2$  ( $\alpha^1\beta_2 + \alpha^2\beta_2$ ) level ranged from 1.77 to 3.40% (mean  $\pm$  SE,  $2.52 \pm 0.05\%$ ). Hb F ranged from 0.43 to 1.80% (mean  $\pm$  SE,  $0.96 \pm 0.05\%$ ). Normal serum iron levels were in general observed. In these subjects the red cell indi-

detected. Globin chain electrophoresis indicated the presence of slow-moving abnormal  $\alpha$ -chains, the subsequent structural characterization of which indicated that it was identical to  $\alpha^{\text{Hasharon}}$ -chain [ $\alpha$  47 (CD5) Asp  $\rightarrow$  His], as previously described [1]. Structural analysis was always carried out at least on one carrier from each family.

### Discussion

The hematological and biosynthetic evidences in the Hb Hasharon north Italian carriers allow us to postulate the existence of an  $\alpha$ -thalassemia-2 determinant linked to the  $\alpha$ -gene carrying the structural abnormality, i.e., of the  $\alpha^{\text{A}}\alpha^{\text{H}}/\alpha^{\text{H}}\alpha^{\text{H}}$  genic arrangement. *Fiorelli et al.* [6] have reported a subject in which both the Hb Hasharon and  $\alpha$ -thalassemia-1 determinants were associated in *trans* and no detectable synthesis of Hb A was observed, thus providing direct support for this hypothesis. Later *Pich et al.* [19] studied another case with analogous features. Whether or not the  $\alpha^{\text{A}}$ -chain gene in *cis* to the  $\alpha^{\text{H}}$ -gene is physically present, it cannot be inferred by the data available to date. In fact, recently the existence of non-deletion  $\alpha$ -thalassemia determinants has been demonstrated [5 10 17] these determinants probably occur with a certain frequency also among non-Asian populations and, in particular in Italy [17 A Cao personal commun., 1979].

The occurrence of Hb Hasharon in the Ashkenazy carriers at significantly lower levels than in Italian carriers allows to postulate the existence of two different haplotypes carrying the same mutation, i.e.,  $\alpha^{\text{A}}\alpha^{\text{H}}$  in Ashkenazy carriers and  $\alpha^{\text{A}}\alpha^{\text{H}}$  in Italian carriers. Indeed, the lack of accurate hematological and biosynthetic studies on

the Ashkenazy carriers does not allow to conclude for a different genic pattern with respect to that of the Italian carriers, though it seems very likely.

The existence of an  $\alpha$ -thalassemia-2 determinant in *cis* to the  $\alpha^{\text{H}}$  locus would account for the surprisingly high frequency of Hb Hasharon trait observed in the northern moiety of the Polesine region, which was previously reported to be about 0.8% [1]. Our more recent estimate deriving from a survey of 10,545 individuals in the province of Rovigo indicates an incidence of 0.23%, which appears to be more accurate. On the contrary other Hb variants remain uncommon, not very much above the estimated mutation rate. Such a high incidence is difficult to be explained, even by assuming a high rate of endogamy due to the past geographical isolation of this particular population. Until to a recent past, the Polesine region has been a district severely afflicted with malaria, the endemicity of which is thought to have been the factor of major importance in determining the very high incidence of  $\beta$ -thalassemia observed in this region [20], and it seems reasonable to assume that even heterozygous  $\alpha$ -thalassemia could have been favored as well. Thus, the  $\alpha^{\text{H}}$  locus could have had a selective advantage because of its linkage to an  $\alpha$ -thalassemia-2 determinant. The selection operated by malaria on  $\alpha$ -thalassemia is in fact well documented by the high incidence of both  $\alpha$ -thalassemia-1 and  $\alpha$ -thalassemia-2 in southeastern Asian population [21] an extreme example of this effect being the virtual genetic fixation of the  $\alpha$ -thalassemia-2 haplotype in the population of Karkar island (New Guinea) studied by *Old et al.* [15].

As far as the origin of Hb Hasharon in northern Italy is concerned, two main hypotheses may be put forward: (1) Introduc-

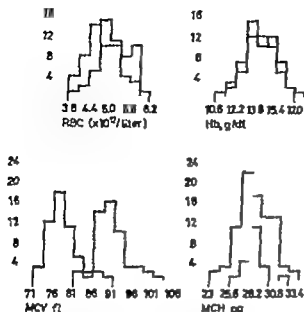


Fig. 2. Histogram representation of the values of RBC indices in the 53 Hb Hasharon heterozygous carriers (—) and in normal controls (---)

$\alpha$ -thalassaemia determinant. In order to provide experimental evidence for this suggestion biosynthetic studies were carried out on blood samples from 17 Hb Hasharon carriers taken at random. The overall  $\alpha/\beta$  chain synthesis ratios ranged from 0.72 to 0.89 as total counts per chain (mean  $\pm$  SE,  $0.81 \pm 0.01$ ) normal range in our laboratory  $0.97 \pm 0.03$  indicating the occurrence of a slight  $\alpha$ -chain deficit consistent with the presence of an  $\alpha$ -thalassaemia-2 determinant (i.e., the presence of 3 out of 4 active  $\alpha$ -chain loci). The mean percentage of Hb Hasharon (i.e., about one third of the total Hb) is compatible with this hypothesis. The  $\alpha^{\text{HbH}}/\text{total } \alpha\text{-chain}$  synthesis ratios ( $0.28 \pm 0.01$ ) are in close agreement with the percentage of abnormal Hb in the hemolysates, and indicate that about one third of the overall  $\alpha$ -chain synthesis is to be ascribed to the mutated  $\alpha$ -gene. The distribution of the Hb Hasharon percent values is

shown in figure 1 together with the distributions of the absolute quantities of  $\alpha^{\text{HbH}}$  and  $\alpha^{\text{L}}$ -chains calculated from the MCH values and expressed as mean pg/cell. The mean content of  $\alpha^{\text{HbH}}$ -chain was  $3.99 \pm 0.04$  pg/cell whereas that of  $\alpha^{\text{L}}$ -chain was  $9.78 \pm 0.09$  pg/cell.

An evident exception is represented by the single 23.7% Hb Hasharon carrier (marked by an asterisk in figure 1), who showed an overall  $\alpha/\beta$  chain synthesis ratio of 1.07. These data seem rather to suggest an arrangement of 4 active  $\alpha$ -chain genes. Nevertheless the red cell indices of the 13-year-old female (RBC,  $4.88 \times 10^{12}/\text{liter}$ ; MCV 78 fl; MCH, 28.1 pg) do not significantly differ from those observed in the other carriers, in contrast to what is expected according to this hypothesis. Although the occurrence of 4 active  $\alpha$ -chain genes appears likely no definitive conclusion can be drawn from the available data.

As far as the double heterozygotes for Hb Hasharon and  $\beta$ -thalassaemia are concerned, they showed RBC indices and morphology alterations typical of  $\beta$ -thalassaemia trait [RBC,  $(5.69 \pm 0.19) \times 10^{12}/\text{liter}$ ; Hb,  $2.93 \pm 0.29$  g/dl; MCV  $68 \pm 0.98$  fl; MCH,  $22.57 \pm 0.32$  pg]. The percentage of Hb Hasharon was  $13.99 \pm 0.42\%$ . Hb  $A_2$  and Hb  $H_{\text{HbH}}$  and Hb F formed  $4.85 \pm 0.15\%$  and  $1.81 \pm 0.49\%$  of the total Hb respectively. The mean  $\alpha^{\text{HbH}}$ -chain content was  $1.58 \pm 0.06$  pg/cell, whereas the  $\alpha^{\text{L}}$ -chain content was  $9.71 \pm 0.13$  pg/cell.

#### Structural Studies

Cellulose acetate electrophoresis of the hemolysates showed a slow-moving Hb S-like Hb band. A minor Hb component (presumably Hb  $H_{\text{HbH}}$ ), migrating more slowly to the anode than Hb  $A_2$ , was also

detected. Globin chain electrophoresis indicated the presence of slow-moving abnormal  $\alpha$ -chains, the subsequent structural characterization of which indicated that it was identical to  $\alpha^{\text{Hasharon}}$ -chain [ 47 (CD5) Asp  $\rightarrow$  His] as previously described [1]. Structural analysis was always carried out at least on one carrier from each family.

### Discussion

The hematological and biosynthetic evidences in the Hb Hasharon north Italian carriers allow to postulate the existence of an  $\alpha$ -thalassemia-2 determinant linked to the  $\alpha$ -gene carrying the structural abnormality i.e., of the  $\alpha^{\text{A}}/\alpha^{\text{Hasharon}}$  genic arrangement. Florelli *et al.* [6] have reported a subject in which both the Hb Hasharon and  $\alpha$ -thalassemia-1 determinants were associated *in trans* and no detectable synthesis of Hb A was observed, thus providing direct support for this hypothesis. Later Pich *et al.* [19] studied another case with analogous features. Whether or not the  $\alpha^{\text{A}}$ -chain gene in cis to the  $\alpha^{\text{Hasharon}}$ -gene is physically present, it cannot be inferred by the data available to date. In fact, recently the existence of non-deletion  $\alpha$ -thalassemia determinants has been demonstrated [5, 10, 17] these determinants probably occur with a certain frequency also among non-Asian populations and, in particular in Italy [17, A Cao personal commun., 1979].

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The existence of an  $\alpha$ -thalassemia-2 determinant in cis to the  $\alpha^{\text{Hasharon}}$  locus would account for the surprisingly high frequency of Hb Hasharon trait observed in the northern moiety of the Polesine region, which was previously reported to be about 0.8% [1]. Our more recent estimate deriving from a survey of 10,545 individuals in the province of Rovigo indicates an incidence of 0.23 %, which appears to be more accurate. On the contrary other Hb variants remain uncommon, not very much above the estimated mutation rate. Such a high incidence is difficult to be explained, even by assuming a high rate of endogamy due to the past geographical isolation of this particular population. Until to a recent past, the Polesine region has been a district severely afflicted with malaria, the endemicity of which is thought to have been the factor of major importance in determining the very high incidence of  $\beta$ -thalassemia observed in this region [20] and it seems reasonable to assume that even heterozygous  $\alpha$ -thalassemia could have been favored as well. Thus, the  $\alpha^{\text{Hasharon}}$  locus could have had a selective advantage because of its linkage to an  $\alpha$ -thalassemia-2 determinant. The selection operated by malaria on  $\alpha$ -thalassemia is in fact well documented by the high incidence of both  $\alpha$ -thalassemia-1 and  $\alpha$ -thalassemia-2 in southeastern Asian population [21], an extreme example of this effect being the virtual genetic fixation of the  $\alpha$ -thalassemia-2 haplotype in the population of Karkar island (New Guinea) studied by Old *et al.* [15].

As far as the origin of Hb Hasharon in northern Italy is concerned, two main hypotheses may be put forward: (1) introduc-



tion of the  $\alpha^{\text{Hb}^{\text{Hb}}}$  mutation by the Ashkenazy carriers settled in Venice at the beginning of the Modern Age followed either by a crossover even between the  $\alpha^{\text{A}}\alpha^{\text{Hb}^{\text{Hb}}}$ -haplotype most probably present in Ashkenazy carriers and an  $\alpha^{\text{A}}$ -haplotype to originate the  $\alpha^{\text{Hb}^{\text{Hb}}}$  pattern (although the minimal inter gene distance between the two linked  $\alpha$ -loci of 3.1 kilobases as measured by Orkin [16] argues against the likelihood of a crossover event between the two  $\alpha$ -genes) or by an unequal crossover with the loss of an entire  $\alpha$ -gene (ii) spontaneous mutation occurred either on an  $\alpha^{\text{A}}$ -haplotype or in principle, on a normal haplotype followed by the loss of an  $\alpha$ -gene caused by the mechanisms indicated above. At present, neither of these hypotheses can be substantiated by any evidence.

The Hb Hasharon trait/ $\beta$ -thalassemia trait combination is characterized by a significant decrease of Hb Hasharon percent age up to about 14%. It is noteworthy that the decrease of the mean cell Hb typical of  $\beta$ -thalassemia is beared almost exclusively by the  $\alpha^{\text{Hb}^{\text{Hb}}}$ -chains, as the absolute quantity of  $\alpha^{\text{A}}$ -chains in these individuals ( $9.71 \pm 0.13$  pg/cell) does not significantly differ from that of non- $\alpha$ -thalassemic abnormal Hb carriers (i.e.,  $9.78 \pm 0.09$  pg/cell). Conconi *et al* [3] demonstrated that the relative  $\alpha^{\text{Hb}^{\text{Hb}}}$  synthesis in Hb Hasharon/ $\beta$ -thalassemia carriers is comparable to that of non-thalassemic carriers, indicating the decreased amount of Hb Hasharon to be originated by a post-translation control mechanism.

The concomitant presence of an  $\alpha$ -thalassemia-2 trait in the Hb Hasharon/ $\beta$ -thalassemia combination is expected to reduce the overall chain imbalance and consequently to ameliorate, to a certain extent, the hematological picture typical of  $\beta$ -thal-

assemia. Indeed, this seems to be the case, but we do not have a sufficient number of doubly heterozygous subjects to perform a statistically significant comparison with simple  $\beta$ -thalassemia carriers, and biosynthetic data on these subjects are at present unavailable.

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## Isopycnic Centrifugation of Human, Pigeon and Guinea Pig Erythrocytes

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Department of Chemistry University of Ibadan, and Department of Chemical Pathology University College Hospital, Ibadan

**Key Words.** Isopycnic centrifugation Human haemoglobin genotypes Sick-cell erythrocytes

**Abstract.** The buoyant densities of human erythrocytes with normal (HbAA) and abnormal heterozygous haemoglobins (HbSS, HbAS HbSC) as well as erythrocytes from guinea pig and pigeon were determined by centrifugation in discontinuous density gradients of metrizamide. Guinea pig, pigeon and human erythrocytes from normal human adult (HbAA) and from heterozygote HbAS were found to have the same density of 1.1381 g/cm<sup>3</sup> while erythrocytes from a sickle-cell patient (HbS) and from a heterozygote HbSC, HbSS and HbSC, which are known to have a higher lipid content than isovolumic normal cells, had a lower density of 1.1261 g/cm<sup>3</sup>. When centrifugation was carried out under non-equilibrium conditions (rate zonal) several sedimentation bands were obtained from each erythrocyte preparation. Differences in the sedimentation rates were unrelated to the haemoglobin composition of the erythrocytes. It is suggested that the lower density of the erythrocytes associated with sickle-cell disease may have significant consequences in the circulation of such cells and in the clinical sequence of the sickling process.

### Introduction

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The present studies were based on the premise that differences in the lipid contents of the various erythrocyte cell types could result in different buoyant densities of the cells. This communication reports the use of metrizamide [2-(3-acetamido-5-N-methylacetamido-2, 4,6-trifluoro-benzamido)-2-deoxy-D-glucose] a recently introduced

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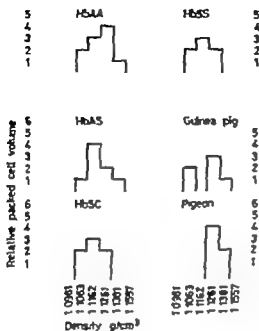


Fig. 1 Sedimentation patterns of human erythrocytes with HbAA, HbAS, HbSC and HbSS and of guinea pig and pigeon erythrocytes in density gradients of metrizamide (20–35% w/v in Krebs-Ringer buffer). Gradients were centrifuged at 201 g (1,000 rpm) for 5 min at 4 °C.

Figure 1 shows the sedimentation patterns obtained on centrifugation under non equilibrium conditions of 201 g for 5 min. On centrifugation of the erythrocytes under this condition several distinct banding patterns were obtained. The packed cell band thickness was determined and indicated as shown in figure 1 as relative ratio of the band volume. The major cell band in the blood of human individuals with homozygous HbA erythrocyte corresponded to 27.5% metrizamide layer.

Identical equilibrium densities and sedimentation patterns were obtained from 12 donors with HbAA and 5 donors with HbAS erythrocytes. Also erythrocytes from 6 HbSS and from 5 HbSC patients behaved in identical manner. Guinea pig erythrocytes

gave three sedimentation bands while the pigeon erythrocytes yielded two bands.

There were no patients with homozygous HbCC disease available during the course of the study.

Electrophoresis of haemoglobin solutions, obtained from each of the sedimentation bands in the heterozygous human Hb A+S and S+C blood samples, showed that each band contained the constituent haemoglobins in proportions approximately equal to those of unseparated samples.

## Discussion

The buoyant density of the normal erythrocyte observed in this study is in agreement with the value observed by other workers using a different method [6]. Further, the results of the present study have indicated some differences in the buoyant densities of human erythrocytes and shown, therefore, that erythrocytes in homozygous HbS and heterozygous HbS+C haemoglobinopathies are less dense than erythrocytes with homozygous HbA or heterozygous HbA+S which were found to have the same buoyant densities.

The significant difference in the densities of the erythrocytes of homozygous HbS and heterozygous HbS+C, both of which are known to undergo the sickling process, may be pathological and may be explicable in terms of the higher lipid content of the erythrocytes in these disease conditions [A 5–9]. Furthermore, this reduction in density of the erythrocytes associated with sickle-cell disease may make significant alterations to the rheological properties of the erythrocytes in the cardio-vascular circulation and contribute to the clinical sequelae of the sickling process.

When the density gradient studies were conducted under conditions which yielded separation on the basis of sedimentation rates, several distinct bands were obtained with each of the human erythrocytes. It is of interest in this respect that erythrocytes of the guinea pig and of the nucleated pigeon gave fewer sedimentation bands although they have the same buoyant density as normal human erythrocytes.

In general, the sedimentation rate of a particle in a density gradient medium is dependent not only on its density but also on the ratio of mass to frictional force as well as hydration properties which will vary with changing osmotic environment down the gradient [8]. The factors responsible for the different sedimentation-rate patterns obtained from the different erythrocytes, which indicated that each blood sample consisted of heterogeneous erythrocyte populations, are not fully understood at present but may however reflect the heterogeneity in the ages of the red cells. Ageing has been shown to decrease the hydration state of red cells [1], and other workers have shown age-dependent alterations in the pattern of sedimentation of erythrocytes [2, 7]. Thus, the buoyant density of the red cell appears to be independent of cell age although the sedimentation rate may be age-dependent. The results also suggest that the sedimentation rates are not dependent on the haemoglobin genotype since in the heterozygote states (HbA+S and HbS+C) the proportions of the constituent haemoglobins were found to be the same in each sedimentation band as in the whole, uncentrifuged sample.

There are two types of expected distributions of haemoglobins in heterozygotes: either the two haemoglobin types are all contained in the same erythrocyte, in which case the haemoglobin composition of the in-

dividual cell would reflect the pattern of composition of the whole blood or the different erythrocyte contains only one of the two types of haemoglobin of the heterozygote thus giving rise to two cell population types, each containing a different haemoglobin. In the latter case the pattern of composition of the haemoglobin would be a result of the relative composition of the two cell population types in each sedimentation band. Since previous work [2] had shown that the pattern of sedimentation rates reflects the age distribution of these cells and since we have now shown that in heterozygotes the haemoglobin composition in each band is the same as in unspun sample, it is plausible that the erythrocytes of different ages that occur in each band of the sedimentation-rate pattern contain both haemoglobins of the heterozygous HbA+S and HbS+C.

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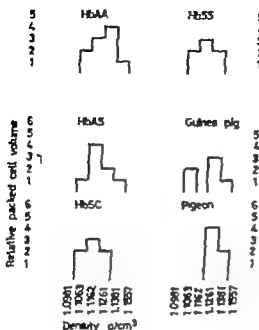


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## Analysis of 75 Cases of Acute Myeloid Leukaemia Classified According to the FAB Classification

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**Key Words.** Acute myeloid leukaemia. Classification

**Abstract.** 75 cases of acute myeloid leukaemia were retrospectively classified according to the French-American-British (FAB) classification. It was found that: (1) the proposed classification was easily applicable and reproducible (2)  $M_1$  subtype was the most frequent (48%) (3) splenomegaly was absent in  $M_1$  subtype, and (4) in  $M_2$  subtype, a considerable number of cases presented with normal neutrophil and platelet counts.

The distinction between acute lymphoblastic leukaemia and acute non-lymphoblastic (myeloid) leukaemia (ANLL) is achieved with certainty by simple morphological and histochemical criteria practically in all cases. This is of importance, as prognosis and treatment in the two types are different.

There have been various attempts to classify the two types further into subtypes. The most recent classification, known as the FAB classification, was established by a group of seven French, American and British haematologists [1]. According to this classification, based on conventional morphological and cytochemical criteria, six subtypes of ANLL are recognised, namely myeloblastic leukaemia without maturation ( $M_1$ ), myeloblastic leukaemia with maturation ( $M_2$ ), hypergranular promyelocytic leukaemia ( $M_3$ ), myelomonocytic leukaemia ( $M_4$ ), monocytic leukaemia ( $M_5$ ) and erythroleukaemia ( $M_6$ ).

In the present study we have attempted,

retrospectively to classify our patients with ANLL according to the FAB classification in order to assess its value.

### Materials and Methods

75 cases of ANLL studied in our unit during 1976-1978 are included in this study. The blood and bone marrow smears of all cases were reviewed independently by three haematologists (ThE, NS, CO) and classified into one of the six subgroups according to the criteria described by the FAB group. In cases of disagreement, the slides were reviewed and discussed by the three examiners.

### Results

There was a complete agreement between the three independent examiners in 70 cases. In 5 cases there was initial disagreement, but when the slides were reviewed and discussed, agreement was achieved in all cases.

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### Frequency of the Six Subtypes Age and Sex Distribution (table I)

Among the six subtypes of ANLL,  $M_1$  is the most frequent (48%) followed by  $M_1$  (16%). The other four subtypes presented approximately with the same proportion (8–10.7%). Among the 75 patients there were 48 men and 27 women with a M/F ratio of 1.7. As far as the age is concerned, a notable finding was the preponderance of old patients in  $M_1$  (median 75 years) and the rather young patients in  $M_2$  (median 34 years).

### Clinical Data

Table I gives the frequency of the main symptoms and signs in the various subtypes.

The number of cases is too small to draw any conclusions. It is interesting to note, however, that splenomegaly was absent in all cases of subtype  $M_1$ .

### Blood Findings

The blood findings at the time of diagnosis are presented in table II. The most interesting finding was the presence of normal neutrophil and platelet counts in a considerable number of subtype  $M_1$  patients.

### Bone Marrow Findings

The main differences in the bone marrow findings are included in the criteria used for the classification. Bone marrow aspiration revealed a hypercellular bone mar-

Table I Distribution of the various subtypes of acute myeloid leukaemia together with sex, age, symptoms and signs at the time of diagnosis

Leukaemia type	$M_1$	$M_2$	$M_3$	M	$M_4$	$M_5$
Number of patients	1	36	6	7 <sup>a</sup>	8 <sup>(<math>M_4A</math> 3 <math>M</math> B 5)</sup>	6
Percentage	16	48	8	9.3	10.7	8
Age (median-range), years	58.5 (25–80)	39 (19–84)	34 (18–70)	60 (35–78)	50.5 (40–67)	75 (74–90)
Males/Females	9/3	1/15	4/2	6/1	4/4	4 <sup>b</sup>
Symptoms						
Fatigue	8	28	2	6	7	5
Fever without infection	6	8	0	2	3	1
Infection	3	22	1	3	3	2
Haemorrhage	3	10	5	5	5	0
Physical findings						
Pallor	1	34	6	■	8	6
Purpura	3	8	4	3	5	0
Splenomegaly	0	9	2	1	3	1
Hepatomegaly	1	8	1	4	3	0
Lymphadenopathy	2	6	0	2	2	■
Gum hypertrophy	0	3	0	3	3	0
Skin involvement	0	0	0	0	1	0

<sup>a</sup> 4 cases were initially diagnosed by one examiner (ThE) as  $M_4$ .  
<sup>b</sup> 1 case was initially diagnosed by one examiner (NS) as  $M_4$ .

Table II. Blood findings at the time of diagnosis of the various types of acute myeloid leukaemia

	M1 (n = 17)	M2 (n = 36)	M3 (n = 6)	M4 (n = 8)	M5 (n = 8)	M6 (n = 10)
Hb > 10 g/dl	1	14	1	5	3	1
Hb < 10 g/dl	11	22	5	2	5	5
Total leucocyte count/litre						
< 4 $10^9$	6	13	2	0	0	2
4.0-10 $10^9$	2	5	1	2	1	3
10-50.0 $10^9$	3	10	3	4	3	1
> 5 $10^9$	1	8	0	1	4	0
Neutrophil count, litre						
< 10 $10^9$	4	14	2	3	2	2
10-40 $10^9$	8	14	4	4	3	4
> 40 $10^9$	0	8	0	0	3	0
Blast count/litre						
< 1.0 $10^9$	4	9	1	1	1	3
1.0-5.0 $10^9$	3	11	1	1	2	2
5.0-50.0 $10^9$	2	13	4	4	5	1
> 50.0 $10^9$	1	3	0	1	0	0
Platelets/litre						
< 20.0 $10^9$	9	11	4	3	4	3
20.0-80.0 $10^9$	3	13	1	1	4	2
80.0-150.0 $10^9$	0	10	1	3	0	1
> 150.0 $10^9$	0	2	0	0	0	0

— = Number of patients.

row with heavy blast infiltration in all cases.

Granulopoietic, erythropoietic and megakaryocytic components were much decreased except in some cases of  $M_2$ . Auer rods were found almost with the same frequency in subtypes  $M_1$ ,  $M_2$  and  $M_3$  (20-25%), whereas in subtype  $M_4$  the proportion was higher (50%). We could not find Auer rods in subtypes  $M_5$  and  $M_6$ . The peroxidase stain was less intensively positive in  $M_4$  cases. The cases with  $M_1$  subtype showed a pattern of scattered small granules and in 1 case no activity was found.

## Discussion

The great range of morphological variation of ANLL and the lack of a uniform classification prompted a French-American-British group of haematologists to propose a new classification [1]. However the validity of this classification still remains unsettled. In the present study we tried to assess the merits of this classification. It was found that it is easily applicable and reproducible. There was initial disagreement between the independent examiners in only 5

of the 75 cases which was settled when the slides had been reviewed and discussed.

Table I shows that the myeloid types of leukaemia  $M_1$ ,  $M_2$  and  $M_3$  were far more frequent (72%) than the leukaemia with partial or total monocytic differentiation ( $M_4$ ,  $M_5$ ). The most frequent group was the  $M_2$  group. The relative frequency of the various types differs considerably from one series to another. Thus, in the MRC report [6] and in others [4-7] the monocytic and myelomonocytic leukaemias were more frequent. This perhaps could be attributed to different criteria used for the classification as the other reports are not based on the FAB classification. In a recent report [5] dealing with the classification of childhood leukaemias according to the FAB classification it appears that  $M_1$  was the most frequent type.

Table I gives the frequency of the main symptoms and signs in the various subtypes. The number of cases is too small to draw any conclusions. It is interesting to note, however, that splenomegaly was absent in all cases of  $M_1$  subtype. In the MRC report [6] based on Galton and Dacie's [3] classification, splenomegaly was found in 39% of the cases of  $M_1$  subtype which is similar but not identical with the  $M_1$  subtype of the FAB classification. In the  $M_2$  type a considerable number of patients presented with normal or increased neutrophil counts and moderately low or normal platelet counts. These findings probably reflect more complete differentiation. In contrast, in the  $M_3$  type, which exhibits the least differentiation, thrombocytopenia was prominent. The frequency of Auer rods in  $M_1$ ,  $M_2$  and  $M_3$  ranged from 20 to 25%. In  $M_4$ , the frequency was higher (50%). We could not find Auer rods in  $M_5$  and  $M_6$ . The number of cases is too small to draw any definite con-

clusions. However, the presence of Auer rods was more prominent in  $M_4$  as has already been reported [1, 2].

Our study which was based on a relatively small number of patients with ANLL, indicated that FAB morphological classification is workable. Further studies on the proposed classification may prove useful in the management of the patients with ANLL.

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## Isoenzyme Variants of Nonspecific Esterases in Chronic Myelocytic Leukemia and in Blastic Crisis

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**Key Words.** Blastic crisis · Chronic myelocytic leukemia · Isoenzymes · Nonspecific esterases

**Abstract.** The isoenzymes of nonspecific esterases have been examined with polyacrylamide gel electrophoresis in 10 patients with chronic myelocytic leukemia (CML) and 10 with blastic crisis (BC) in respect to normal subjects. In patients with active CML the recurrent loss of fast bands was detected, while in patients in remission the isoenzyme variants are similar to those found in normal subjects. Different isoenzyme variants which are similar to those found in acute leukemia were detected in patients with BC of different morphological type with the exception of BC of megakaryoblastic type. The possible meaning of such variants in relation to an early identification of malignant proliferating clones, is discussed.

Chronic myelocytic leukemia (CML) is a relatively common disease and one of the few malignant diseases in which definite aspects of pathophysiology are known [10]. In recent years the development of culture techniques, particularly using diffusion chambers, improved the knowledge of factors controlling proliferation and maturation of granulocyte precursors in both normal and CML patients.

It is known that some degree of regulation of hemopoiesis persists in CML at least until blastic crisis (BC) takes place. Chikkappa *et al.* [3] demonstrated by diffusion chamber technique that CML cells in cul-

ture not only mature to neutrophils but even develop alkaline phosphatase (LAP) activity. These data are apparently in contrast with the low levels of the enzyme which is found in the patient's circulating granulocytes.

The research of isoenzyme variants as possible markers for leukemic cells is becoming more and more important. The cytochemical studies sometimes do not allow a whole phenotypical characterization of the blasts of BC. Nonspecific esterases (NSE) as  $\alpha$ -naphthyl acetate and/or  $\alpha$ -naphthyl butyrate esterases are the more studied among the lysosomal enzymes, because they are

present in different cellular clones, lymphocyte [2] and granulocyte precursors [6]

This report concerns a study of NSE isoenzyme variants in CML and in BC of CML.

## Materials and Methods

20 patients with CML in the chronic phase and in BC were studied. In the chronic phase the patients cells were examined (a) before therapy (b) during the treatment, when promyelocytes and band cells were predominant and (c) during treatment with predominance of PMN. All the patients, treated following a protocol published elsewhere [12], had Ph chromosome anomaly and reduced activity of LAP.

The 10 patients in BC had a morphologically different cell proliferation. The cells appeared to be undifferentiated in one instance, myeloblastic in four monoblastic in three and megakaryoblastic in two. Diagnosis of various types of BC, using

accepted morphological criteria [11], was made on the basis of Giemsa, peroxidase, periodic acid-Schiff, specific and nonspecific esterase stained films of peripheral blood and bone marrow. 5 healthy donors were also studied.

**Esterases Extraction and Assay** Buffy coat from EDTA peripheral blood and/or bone marrow was suspended in 0.1% cetyltrimethylammonium bromide (Merck, Darmstadt) to a final concentration of  $10 \times 10^6$  cells/ml. The cells were lysed with six cycles of freezing and thawing ( $-80^\circ\text{C}$ ) and stored at  $-80^\circ\text{C}$  before assay [11]. The electrophoretic studies were performed within 2 weeks. The cellular lysates (75  $\mu\text{l}$ ) were electrophoresed in 7.5% polyacrylamide gel, pH 8.3, in a conventional Canaco Apparatus [4]. The run was carried out at room temperature for 60 min. After the run, gels were washed in M/15 phosphate buffer pH 6.3 at  $4^\circ\text{C}$  for 30 min and stained. Staining for NSE was performed at the same pH using, as substrate,  $\alpha$ -naphthyl acetate and  $\alpha$ -naphthyl butyrate for 1 and 6 h, respectively [9]. Separate gels were stained in NSE incubation media containing sodium fluoride [9].

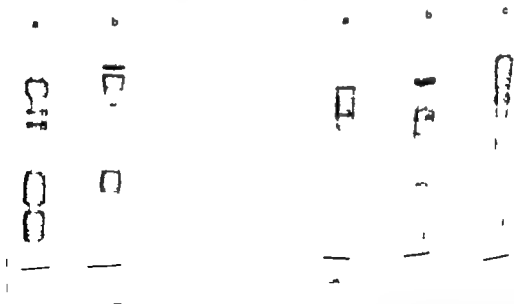


Fig. 1 NSE in normals. Cetyltrimethyl ammonium bromide extracts. a  $\alpha$ -Naphthyl acetate. b  $\alpha$ -Naphthyl butyrate.

Fig. 2. NSE in CML. Cetyltrimethyl ammonium bromide extracts. a Patients before therapy b Patients in therapy with predominance of promyelocytes and myelocytes. c Patients in therapy with PMN predominance.  $\alpha$ -Naphthyl acetate.

# Results

## Normal Controls

In specimens from 5 normal subjects (high PMN counts) 7 light bands, 4 slow (orthodical) and 3 fast (anodical) in comparison to bromophenol blue dye front, were observed (fig. 1)

## Chronic Phase Patients

In chronic phase, lysates from PMN and other cells (granuloblastic precursors, mainly mature) stained with both substrates showed a reduced number of bands, due to the loss of 1-3 anodical bands. In particular using  $\alpha$ -naphthyl acetate substrate all the anodal bands disappeared in lysates from patients before and during treatment (fig. 2a, b) while, when a PMN predominance was observed, all the bands found in

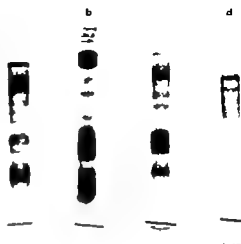


Fig. 4. NSE in BC. Cetyltrimethylammonium bromide extracts. BC monoblastic type. b BC myeloblastic type. BC undifferentiated type. d Megakaryoblastic type.  $\alpha$ -Naphthyl acetate



Fig. 5. NSE in CML. Cetyltrimethylammonium bromide extracts. Patients before therapy. b Patients in therapy with predominance of promyelocytes and myelocytes. Patients therapy with PMN predominance.  $\alpha$ -Naphthyl butyrate

Fig. 6. NSE in BC. Cetyltrimethylammonium bromide extracts. BC monoblastic type. b Myeloblastic type. BC undifferentiated type. d BC megakaryoblastic type.  $\alpha$ -Naphthyl butyrate.

normals were present (fig. 1 2c) Using  $\alpha$ -naphthyl butyrate as substrate, a similar loss of fast bands was observed as to the slow bands, a variable reduction in their number was found (fig. 3)

### *Blastic Crisis*

The gels from monoblastic BC showed 5 slow bands of NSE activity with both substrates. Band No 5 was prominent. In lysates from BC of undifferentiated and myeloblastic type a pattern similar to normal was observed with both substrates. In lysates from BC of megakaryoblastic type, at least 4 slow bands could be detected, using  $\alpha$ -naphthyl acetate, while only 1 slow band was found using  $\alpha$ -naphthyl butyrate. All the anodal bands are lost whatever substrate is used (fig. 4 5)

### **Discussion**

In this study we tried to ascertain if it is possible to show using polyacrylamide gel electrophoresis, the activity deficit of NSE and the possible presence of their anomalous variant in CML.

In agreement with Kass *et al* [8] our results indicate that in patients with CML in remission (c) NSE electrophoretic patterns are similar to those detected in normal subjects. This may confirm that during the remission stage CML granulocytes maintain or reacquire the biochemical and metabolic properties of normal granulocytes [1]. On the other hand in patients with active CML (a and b) the constant loss of 2 or 3 fast bands and a poor differentiation of slow bands has been observed. Therefore, it may be supposed that anodal bands are features of a more mature cellular population or if present, their quantity in myeloblasts pro-

myelocytes and myelocytes is so small that they cannot be detected.

Completely different patterns of NSE activity were observed in patients with BC (fig 4 5) in fact a pattern of at least 6 bands with both substrates was shown. none of these seems peculiar to one cellular type except for band 5 which is more evident in BC of monoblastic type. As these findings are similar to those described in spontaneous acute leukemia with the same morphological type [7 9] it could be supposed that in the granulocytic and monocytic series there is only one system of NSE which is present in a continuum of differentiation.

The electrophoretic patterns of BC, except the megakaryoblastic ones, seem to be in contrast with those of CML in the active stage which is characterized by the loss of anodal bands. This may lead to envisage the possibility of a selection of blastic clones different from those observed at onset of CML. In this context the electrophoretic patterns in the megakaryoblastic lysates, in which the constant loss of anodal bands is similar to the one shown in the granuloblastic precursors of CML at onset, seem of difficult interpretation.

However our results suggest some conclusions (1)  $\alpha$ -naphthyl acetate is more specific as a substrate than butyrate as it shows a greater number of isoenzyme variants (2) precursors of granulocytes during CML show in primitive nondifferentiated forms, the same variants as found in normal granulocytes (3) BC, considered as true acute leukemia presents electrophoretic patterns of NSE similar to those found in acute leukemia of the same morphological type.

The small and nonhomogeneous group of patients we have examined up to now does not allow any definitive conclusion. However a detailed study of single patients

with CBIL, may be useful to detect the anomalous isoenzyme variants which could be a feature of the malignant proliferating clone.

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## Neutrophil Chemiluminescence during the First Month of Life

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**Key Words.** Chemiluminescence Infants Neutrophils

**Abstract.** Postphagocytic chemiluminescence of neutrophils was evaluated in infants throughout the 1st month of life in their mothers and healthy adults to investigate further the defective chemiluminescence of neonatal neutrophils described by earlier studies. Maternal and adult values were similar and served as controls. Abnormalities both of peak chemiluminescence and of the kinetics of light emission were detected in infant neutrophils throughout the 1st month of life. Infant responses were variable, particularly at birth, when neutrophils of some infants performed comparably to those of controls.

Newborn infants have an increased susceptibility to infection and the mortality from infections is higher than in older children. Although a number of abnormalities of the body defense mechanisms have been described in infants, results are variable and controversy exists regarding the relative importance of each defect [5]. Many investigators have found normal or increased rates of oxidative metabolism in neutrophils (N) from human infants. Disagreement exists, however among reports assessing postphagocytic oxidative metabolism by chemiluminescence (Cl). In this assay oxidative metabolism is estimated by the quantity of light that is emitted from phagocytic N as a consequence of complex reactions in which electronically excited molecules are formed.

In patients with chronic granulomatous disease of childhood, recurrent infections are correlated with a complete lack of Cl and the inability to increase N postphagocytic oxidative metabolism.

Abnormalities of both peak Cl (the greatest amount of light emitted following phagocytosis) [2, 7] and of the kinetics of light emission [6, 7] have been reported in N obtained from apparently healthy infants. However others [3] found Cl to be abnormal only in sick infants. N of healthy infants were normal. Because of this controversy we wish to report the results of Cl assays performed using N isolated from venous blood of infants at varying ages throughout the 1st month of life, their mothers and controls.

## Materials and Methods

Studies were approved by the local committee governing human investigations and informed consent as obtained. Venous blood was collected from mothers and their full-term infants within 4 h of birth (20 infants) and at either 1-2 weeks (10 infants) or 4 weeks (12 infants) of age. Adults served as controls. All subjects were healthy and were taking no medications on regular bases. Suspensions containing  $> 90\%$  N and nearly devoid of erythrocytes and platelets were prepared by dextran sedimentation, Ficoll-Hypaque centrifugation and hypotonic lysis. In pilot study infant, mother and control N were found to be comparable. Aliquots of suspensions were deposited on slides by cytocentrifugation and examined after staining Erythrocyte N and platelet N ratios never exceeded 1:1,000 and 1:100. Mature N (segmented nuclear lobes with indentations between lobes being  $< 20^\circ$  of lobe diameter) accounted for  $> 75\%$  of N in all samples. Most remaining cells are N of nearly similar maturity with slightly wider indentations. N were  $> 90\%$  viable by erythrosin B dye exclusion, and  $> 80\%$  phagocytized opsonized zymosan particles during the CI reaction (assessed by examining stained cytocentrifuge slides). Quantitative differences between infants and controls were not apparent by simple visual inspection. Studies to detect the rate of phagocytosis were not performed. The CI technique was as before [6], and phagocytic reactions contained  $2.5 \times 10^4$  N and  $125 \times 10^3$  opsonized zymosan (1:50).

Statistics were performed by Dr. Lew F. Bernstein, Department of Preventative Medicine and Environmental Health. Many experimental conditions influence the CI assay and results can vary greatly [1, 4, 7]. Because of this imprecision, each infant and mother were studied simultaneously with control and the performance of infant and maternal N was related to that of the simultaneous control and expressed as

$$\frac{\text{percent of control} = \frac{\text{infant or mother value}}{\text{simultaneous control value}} \times 100$$

Arithmetic means of the actual peak CI values and of the percent of control were calculated, as were logarithmic means of the former and the significance of differences between means was tested by

Student's *t* test. In addition, infants and their respective mothers were evaluated by paired analysis employing paired *t* and sign tests.

## Results

Postphagocytic peak CI values of infant N expressed as percent of the simultaneous control (fig. 1), were significantly decreased throughout the 1st month of life when compared with their mothers and controls ( $p < 0.01$  at birth,  $p < 0.05$  at 1-2 weeks,  $p < 0.01$  at 4 weeks of age). Mothers and controls were similar ( $p > 0.05$ ). Infant values were particularly variable at birth, and N from several infants (9 of 20) performed comparably to mothers and controls, where

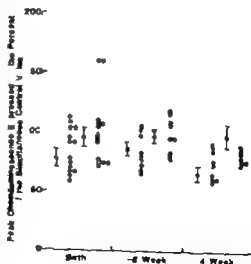


Fig. 1. Relationship of peak CI values of infant (●) and maternal (○) neutrophils when related to the value of control studied simultaneously. Mean infant values are significantly less than controls at all ages studied, while maternal and control values were similar.  $\bar{x}$  = Mean  $\pm$  SEM.

Table 1. Peak CI values (mean  $\pm$  SEM of infants, their mothers and simultaneous controls)

Infant age	Percent of control		Actual values as cps $\times 10^3$		
	Infant	mother	Infant	mother	control
<24 h (20 groups)	79 $\pm$ 7	95 $\pm$ 7	61 $\pm$ 8	79 $\pm$ 10	90 $\pm$ 12
1-2 weeks (10 groups)	86 $\pm$ 6	97 $\pm$ 5	58 $\pm$ 4	64 $\pm$ 5	69 $\pm$ 7
4 weeks (12 groups)	65 $\pm$ 5	98 $\pm$ 9	97 $\pm$ 18	166 $\pm$ 40	163 $\pm$ 58

as they were consistently defective at the age of 4 weeks (fig. 1). The explanation for this variability at birth is unknown. Perhaps the normal values are the consequence of some undefined stimulation that is related to the intrauterine environment or to birth. It elevates CI at birth, but is dissipated by the age of 4 weeks.

Peak CI of infant N was decreased ( $p < 0.05$ ) only at birth when differences between the actual CI peak values (to be distinguished from the percent of control) were

analyzed by logarithmic and geometric means (table 1). Differences were not significant at later ages when analyzed by these techniques, probably a consequence of the small numbers of subjects and great variation. However, significant differences were detected when performances of neonatal N were compared with those of their respective simultaneous controls.

Kinetic abnormalities of the CI response elicited in infant N were reported by some [6, 7] but not all [2] investigators. To define kinetics of light emission more precisely, N were obtained from 16 infants during the first 24 h of life. CI values at 10 min postphagocytosis and at 10-min intervals thereafter were compared with those of the simultaneous controls (fig. 2). Kinetic abnormalities (i.e., differences in CI throughout the response rather than simply different peak CI values) were suggested, since CI of infant N was significantly less at every time studied ( $p < 0.05$  at 10 and 40, and  $p < 0.01$  at 20 and 30 min after addition of opsonized zymosan).

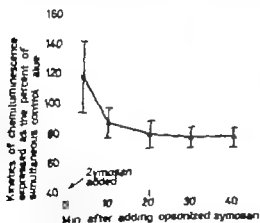


Fig. 2. Comparison of CI values of neutrophils from 16 infants with those of simultaneously studied controls when measured at 10-min intervals after a phagocytic stimulus. Values before phagocytosis (time zero) were similar but infant values at all times after adding opsonized zymosan were significantly less than controls.  $\bar{x}$  = Infant value mean  $\pm$  SEM.

## Discussion

In support of earlier findings [2, 6, 7] postphagocytic CI of N from human infants was decreased at birth. The present data extend earlier observations by documenting

the persistence of this defect in N for at least the first 4 weeks of life. Both peak values and the kinetics of the response were abnormal when compared with the performance of maternal and control N. Maternal and control values did not differ. Although the mechanism(s) responsible is unknown, the defect is probably biochemical and not related to impaired phagocytosis since in fact N, in agreement with others [2] were viable by dye exclusion and capable of particle ingestion. Admittedly however rates of phagocytosis were not measured, and it is impossible to completely exclude this possibility as an explanation for the kinetic differences.

The importance of this abnormality as one of the factors that renders infants susceptible to infections remains to be established. It is clear based on experience with chronic granulomatous disease that a complete lack of CI is a severe N functional defect. The CI response is far from absent in neonatal N. In the present study infant N averaged at least 2/3 of control CI values (table I), and N from some infants performed normally at birth (fig. 1). When data from all reports [2, 3, 6, 7] are reviewed, values from many infants fall into the normal range. Furthermore, the role of CI in N antimicrobial mechanisms is only partly understood. Agreement exists that CI is related to the presence of electronically excited molecules, however it remains to be established whether or not the molecules responsible for light emission are directly involved in N microbicidal activities.

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### Burkitt's Lymphoma Occurring 6 Years after Hodgkin's Disease<sup>1</sup>

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**Key Words.** Burkitt's lymphoma Chemotherapy Hodgkin's disease Radiotherapy

**Abstract.** The occurrence of Burkitt's lymphoma (BL) following Hodgkin's disease (HD) has not yet been reported. A patient treated for HD by chemotherapy-radiotherapy association developed a BL 6 years later: it was demonstrated by cytological, cytochemical, immunological and cytogenetical - translocation t(8;14) - characteristics. Our hypothesis is that, in this case, BL may be the consequence of chemotherapy-radiotherapy association.

#### Introduction

Hematological neoplasia, especially acute leukemias following Hodgkin's disease (HD) have been frequently reported since the development of intensive chemotherapy and/or high voltage radiotherapy [1]. Recently non-Hodgkin's lymphomas (NHL) have also been suspected to be another kind of malignant tumor possibly resulting from chemotherapy-radiotherapy association [2]. We are reporting here the first case of Burkitt lymphoma (BL) following HD cured by chemotherapy-radiotherapy association.

#### Case Report

In October 1971 a 21-year-old white woman was admitted to the Institut de Recherches sur les Maladies du Sang (Hôpital Saint-Louis, Paris) for HD - clinical stage IIA, nodular sclerositis type. She was treated by 3 courses of MOPP association (methylchloroethamine, vincristine, procarbazine, prednisone) followed by mantle irradiation (40 Gy in 4 weeks). She reached complete remission and received 12 courses of monthly injections of vinblastin sulfate. Treatment was stopped in April 1973. She remained healthy for 6 years after completion of treatment. In April 1979 she was readmitted to the Institute. She was 4 weeks pregnant and suffered from dyspnea. Physical examination showed massive right breast involvement by tumour masses with right supraclavicular tension and superficial venous distention. Examination of both supraclavicular tumor and pleural effusion cells showed a massive infiltration by monomorphous blast cells (20-25 µm in diameter). Their cytoplasm was uniformly and deeply basophilic without granules. Most cells showed

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empty cytoplasmic vacuoles (1.2  $\mu$ m in diameter) on the May-Gruenwald-Giemsa smears. The nuclei are round and large with finely clumped chromatin. Many mitotic figures were observed. The blast cells are usually devoid of PAS-positive material. Oil red O staining showed the presence of coarse lipid droplets in the cytoplasmic vacuoles. The acid phosphatase reaction was negative or rarely positive. The peroxidase reaction was negative. According to the FAB classification, the cells were classified as L3 lymphoblasts, Burkitt type [3]. Bone marrow and peripheral blood dissemination were not present. The study of surface immunoglobulin by direct immunofluorescence showed that 97% of the cells had both  $\mu$  and  $\delta$  heavy chains and  $\bar{K}$  light chains. Cytogenetical studies using banding techniques proved t(8;14) translocation in all cells which had divided. EBV-determined nuclear antigen tests were not performed.

These morphological, immunological and cytogenetical findings are presently known as BL or Burkitt-type leukemia characteristics [4, 5]. Moreover such clinical presentation (breast involvement in pregnant women) has already been described [6].

## Discussion

The pathological material of October 1971 was reviewed in July 1979. It showed typical features of HD nodular sclerosis type, thus excluding the possibility of a wrong initial diagnosis. To our knowledge, BL after HD has never been reported. Numerous hypotheses could account for the HD-BL succession. It might be coincidental. BL could be a histological transformation of HD by the therapy (radiotherapy and/or chemotherapy may be mutagenic and contribute to the appearance of BL, the impairment of cellular immunity observed in HD or an immune deficiency state acquired under therapy - or both mechanisms - may contribute to the genesis of BL. This last hypothesis is the most convincing for the

following reasons. Immune abnormalities have been described as associated with African BL [7] other types of B cell malignancies following immunosuppressive therapy of malignant or nonmalignant conditions have been reported [8, 9] recently 5 NHL (most NHL are known to be of B cell origin) have been reported after combined modality therapy for 344 patients suffering from HD [2] the actuarial risk of such an occurrence has been calculated to be 1.3% at 7 years (and 15.2% at 10 years!). Moreover 4 of the 5 patients had an undifferentiated histological type, more or less resembling BL. In the future, with the help of cytological, immunological and cytogenetical methods, there will probably be more diagnoses of Burkitt-type lymphomas as well as of other neoplasias of B cell origin following immunosuppressive treatment.

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## Factor VII Survival Studies in Factor VII Padua Abnormality<sup>1</sup>

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**Key Words.** Factor VII Abnormal factor VII Factor VII survival tissue thromboplastin

**Abstract.** Two patients with factor VII Padua abnormality were studied during prophylactic administration of factor VII concentrates for tooth extractions. The amounts administered were 15.8 and 17.2 units/kg body weight in 30 and 20 min, respectively. The end infusion factor VII activity levels were 55 and 64%, respectively. Survival times of the exogenous components were 6.5 and 6 h, respectively. Satisfactory hemostasis was obtained in both instances.

Factor VII Padua clotting disorder was described by us in 1978 [5]. The defect consists in an abnormality of factor VII activation whereby factor VII activity in affected patients, appears normal if ox brain thromboplastin is used in the assay system whereas it is about 10% of normal if determined with rabbit brain thromboplastins. Human tissue thromboplastins yield intermediate levels.

Nothing was known about the survival times of exogenous factor VII in these patients. The opportunity we had to study two patients with this disorder while they were undergoing prophylactic treatment with factor VII concentrates for tooth extractions prompted this brief report.

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### Patients, Materials and Methods

Patient 1 is a 43-year-old female and the index patient with this disorder who was already extensively studied by us [5]. In preparation for a multiple tooth extraction (three upper molars and three upper incisors) she was treated with 1,000 units of Proconvertin U.M. (Immuno S.p.A., Pisa, Italy) together with 300 ml of fresh plasma, for a total of 1,300 units (15.8 units/kg body weight). Her body weight was 87 kg, the hematocrit was 40, the calculated whole blood mass was 5,900 ml, the plasma volume was 1,500 ml. The infusion of concentrate and plasma lasted 30 min and blood was drawn before, immediately after the infusion and 2.5, 5, 10, 15, 20, 30, 45, 55 and 70 h thereafter.

The second patient belongs to a different family and was also described by us elsewhere [6]. She is a 20-year-old female who received 1,000 units of Proconvertin U.M. (Immuno, Wien) during a 20-min period in preparation of a single tooth extraction (upper left premolar). Her body weight was 58 kg, the hematocrit was 40, the calculated blood mass was 4,000 ml, the plasma volume was



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of the studies carried out dealt with the infusion of large quantities of plasma which made all but impossible to calculate a satisfactory half life. Often, with plasma, only one component of the curve was evident [10]. Furthermore, the presence of bleeding during substitution therapy may have influenced the survival times. This may explain the very short half-lives observed in some instances [10]. In our patients, the bleeding was very slight. In addition, no thyroid dysfunctions or fever, conditions known to alter survival of clotting factors [7-17] were present. Therefore, it may be assumed that the values observed were the real ones.

Taking into account the mildness of the bleeding tendency presented by the propositae and the prompt correction of the clotting defect observed in the present studies, it would seem that the propositae could undergo even major surgery without difficulties.

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2,500 ml. The amount transfused was therefore 17.2 units/kg body weight. Blood samples were drawn before, immediately after the infusion and 2.5, 5, 10, 20, 30, 40 and 65 h thereafter.

Materials and methods have been described in detail elsewhere [3-6]. Factor VII level was assayed as previously reported using a rabbit brain and a lung thromboplastin preparation and factor VII-deficient plasma as substrate [5, 6]. To calculate the exogenous fraction, 9 or 10% were subtracted from each reading for case 1 and case 2, respectively. Such factor VII values represent in fact the endogenous levels of factor VII activity present in the two patients, as determined by rabbit brain and lung thromboplastin [5, 6].

## Results

In the first patient, the factor VII level at the end of the infusion was 55%. Factor VII survival time of the exogenous fraction was 6.5 h (fig 1). In the second patient, the factor VII level at the end of the infusion was 64%. In this case, the half-life of the exogenous fraction was 6 h. Both curves showed two components, a rapid and a slow one. No bleeding was noted after the tooth extractions in either patient. No untoward side-effect was noted after the infusion of the factor VII concentrate. Healing of the

gingival sockets after tooth extraction was as expected.

## Discussion

Survival time in factor VII Paderborn abnormality is about 6 h. The slope of the curves was practically identical in the two patients. The half-life of the patient who received the concentrate together with plasma was slightly longer (6.5 h) and this was probably due to the longer infusion time. In spite of the short half-life of the injected material a satisfactory hemostasis could be achieved without any difficulty. These data indicate that the presence of an abnormal factor VII does not affect the survival time of injected factor VII. This observation is in agreement with *in vitro* data which indicate that these patients lack inhibitors in their plasma [6]. It is interesting to note that similar results have been obtained by us in another abnormality of the prothrombin complex factors, namely factor X Friuli disorder [4]. The survival times observed are well in line with those reported in the literature [1, 2, 8-12]. However, it is worth mentioning that some

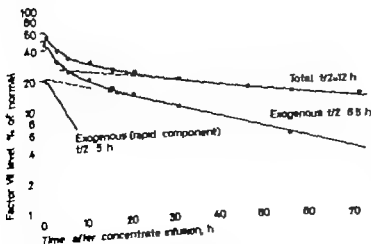


Fig. 1. Survival time in case 1. The patient received 1,000 units of factor VII concentrate together with 300 ml of fresh plasma (15.8 units/kg body weight) in 30 min. The survival time of the exogenous component was 6.5 h. A similar pattern was observed in case 2. The survival time of the exogenous component in this latter case was 6 h.

of the studies carried out dealt with the infusion of large quantities of plasma which made all but impossible to calculate a satisfactory half-life. Often, with plasma, only one component of the curve was evident [10]. Furthermore, the presence of bleeding during substitution therapy may have influenced the survival times. This may explain the very short half-lives observed in some instances [10]. In our patients, the bleeding was very slight. In addition, no thyroid dysfunction or fever conditions known to alter survival of clotting factors [7-17] were present. Therefore, it may be assumed that the values observed were the real ones.

Taking into account the mildness of the bleeding tendency presented by the propositae and the prompt correction of the clotting defect observed in the present studies, it would seem that the propositae could undergo even major surgery without difficulties.

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## Normal Levels of Antithrombin III in Acute Leukemia Complicated by Coagulopathy

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**Key Words.** Antithrombin III · Acute monoblastic leukemia · Coagulopathy

**Abstract.** A patient with acute monoblastic leukemia developed a coagulopathy during chemotherapy. Coagulation studies showed thrombocytopenia, hypofibrinogenemia, high levels of fibrinogen degradation products, a negative protamine test but a normal antithrombin III level. These observations suggest that coagulation abnormalities appearing in acute leukemia are not necessarily due to diffuse intravascular coagulation.

### Introduction

Acute leukemia, especially acute promyelocytic leukemia [5] and acute monoblastic leukemia (AMoL) [7] is frequently complicated by coagulopathy. These coagulation abnormalities have been ascribed to the release of tissue factors with fibrinolytic and proteolytic activity from blasts [10]. Little is known about the level of antithrombin III (AT III), a potent inhibitor of activated coagulation factors, in these situations. The scarce available information seems to indicate that AT III can be normal in these situations [11]. We studied these levels in a patient with AMoL, who developed a coagulopathy while on therapy.

### Case Report

A 37-year-old woman was admitted to our hospital with complaints of increasing fatigue. The major findings on physical examination were proptosis of the left eye, cervical lymphadenopathy, tumors in the mammary glands, and an enlarged liver. Hemoglobin concentration was 93 g/l, packed cell volume 27%, thrombocytes  $95 \times 10^9$ /liter, white cell count  $151 \times 10^9$ /liter with 97% blast forms. A diagnosis of AMoL was made (positive  $\alpha$ -naphthyl-acetate-esterase, elevated serum lysozyme).

Chemotherapy consisting of vincristine 1 mg/m<sup>2</sup> on days 1, 7 and 14 and prednisone 60 mg/m<sup>2</sup> daily was started within 24 h after admission. 5 days after starting chemotherapy spontaneous ecchymoses developed and diffuse bleeding after venipuncture was noticed, partially controlled by platelet transfusion. Severe disturbances in the

Table I. Coagulation studies

	Normal range	Days of chemotherapy									
		1	3	5	6	8	9	10	11	13	17
PT <sup>a</sup> sec	10-12			12.9	16.7	15.4	13.4	11.7	11.7	12.1	12.0
APTT <sup>b</sup> sec	28-35			34.5	32.6	29.8	26.9	27.0	25.2	27.4	26.6
Fibrinogen <sup>c</sup> mg%	200-400			200	175	175	175	130	180	280	240
FDP <sup>d</sup> µg/ml	0-10				320	220	90	90	50	40	0-10
ATIII <sup>e</sup> %	95-190			116		117	109	126	120	127	102
Protamine test <sup>f</sup>	-			-	-						
Blast cells											
10 <sup>6</sup> WBC		151	32					0.21			0
Thrombocytes											
10 <sup>6</sup> /liter	150-140	83	48	22			18	5			12

Activated thromboplastin reagent (Dade)

Automated APTT reagent (General Diagnostics).

Measured chemically with Baret method according to *Laclet and Rhodabarlet* [8].

Thrombo-Welcotest (Wellcome).

Chromogenic substrate (S-2238 Kabi-Diagnostica, Sweden).

Protamine sulfate Organon-On (Holland).

coagulation parameters were found, especially of the level of fibrinogen degradation product (FDP), and the one-stage prothrombin time (PT). Also significant decrease of fibrinogen was found, but the activated partial thromboplastin time (APTT) was normal. Fibrin monomers seemed to be absent as the protamine test was found to be negative on repeated occasions (table I).

## Comments

The appearance of a coagulopathy in acute leukemia was initially ascribed to fibrinolysis. Later diffuse intravascular coagulopathy [6] was considered to be responsible because of hypofibrinogenemia, low factor V VIII and high FDP titers, all of which may reflect the consumption of coagulation factors [2]. One would expect the consequence of 'consumption' to be a de-

crease of AT III [13] a major inhibitor of thrombin and other serine proteases in the blood coagulation system. AT III levels in our patient were normal throughout her clinical course. In a review of leukemia and diffuse intravascular coagulation, *Troblisch and Egbring* [11] also found a normal AT III level. The normal level suggests that this coagulopathy is not caused by diffuse intravascular coagulation leading to consumption of coagulation factors.

The leakage of proteases [4-9] e.g., elastase and chymotrypsin-like proteases, from leukemic blasts may be responsible, and might be intensified by cell-damaging chemotherapy. The release of elastase-like proteases would induce proteolysis of the individual clotting factors and would produce the formation of fibrinogen degradation products. The recognition of this spe-

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### The Congenital Variants of the Prothrombin Complex Factors

I have read with great interest the Editorial on 'Variants of Vitamin K Dependent Coagulation Factors' (Acta haemat. 62: 1 1979).

I would like to add a few comments on factor VII, factor IX and factor X variants.

**Factor VII variants.** There is no doubt that factor VII Padua represents the first factor VII abnormality for which a peculiar activation pattern was surely demonstrated. The discrepancy between rabbit and ox brain thromboplastin clotting time is striking. However I think factor VII Verona also represents a factor VII abnormality which justifies the toponym given to it. The reasons, part of which deal with still unpublished observations, are the following:

(1) The two *propositi* are double heterozygotes for true factor VII deficiency and abnormal factor VII.

(2) The factor VII levels in the two *propositi* are about 20% of normal with rabbit or human brain thromboplastins but about 40% with ox brain thromboplastins. This was not included in the original paper [4] since at that time we failed to recognize it. It was found subsequently after the discovery of factor VII Padua, when additional tests were carried out. However even in the orig-

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(3) Furthermore, factor VII Verona seems to be activated by exposure to glass and/or cold in an abnormally fast manner.

That the interaction between tissue thromboplastins,  $Ca^{++}$  and factor VII is much more complicated than was originally thought is well documented by another abnormality which recently came to our attention, factor VII Padua, [6]. This factor VII shows increased sensitivity to ox brain thromboplastins. Such thromboplastin seems to play a pivotal role in detecting factor VII abnormalities. In some cases, there is no sensitivity at all (Padua), in others, there is a partial sensitivity (Verona) and in still others, on the contrary an exaggerated sensitivity (Padua<sub>2</sub>) to ox brain thromboplastin. Needless to say that many additional variants may exist. It is interesting to note that pig brain thromboplastins behave in a way similar to ox brain preparations [5, 6]. In the past, ox brain thromboplastin was claimed to be particularly sensitive to factor X and/or to the so-called, but never proven, coumarin-induced inhibitors [8]. Probably the main peculiarity of this thromboplastin is its close and multifaceted relationship

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cific factor X assay was included in the study

I fully agree with the recommendations to use several 'activating' systems in the investigation of congenital clotting defects. Unfortunately the methods pertaining to  $Ca^{++}$  binding capacity adsorption to phospholipids and proteolytic digestion are not widely known yet and are still in need of a satisfactory standardization.

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Table I. Main features of surely proven factor X variants

Index patient	Factor X activity			Factor X antigen	Proposed nomenclature
	tissue thromboplastin	RVV cephalin	cephalin		
Mr Stuart (classical factor X deficiency)	absent	absent	absent	absent	X
Miss Prower	low	low	low	normal	X <sub>+</sub>
Mrs. Minin (factor X Friuli)	low	normal	low	normal	X Friuli

with factor VII together with a marked sensitivity to the hemophilia B<sub>u</sub> defect. Since all the families with the three defects studied by us live in the north-eastern part of Italy namely in an area about the size of Holland, I suspect that the different abnormalities are just an expression of a closely related molecular change.

The following tentative classification for the factor VII defects may be proposed (1) factor VII<sub>-</sub> (classical factor VII deficiency) (2) factor VII<sub>l</sub> (patients with low activity but normal CRM) (3) factor VII reduced (patients with low activity and variable CRM which however is always higher than the clotting counterpart but never normal) (4) factor VII Padua (rabbit brain) (5) factor VII Verona (rabbit and human brain) (6) factor VII Padua, (ox brain)

Indication of the thromboplastin that yields the lowest factor VII activity level is absolutely necessary in cases with abnormal activation patterns, lest confusion is generated with simple factor VII or factor VII reduced variants. It is interesting to note that variants with abnormal activation patterns had not been predicted in the genetic nomenclature proposed by Graham *et al* [7]

**Factor IX variants** The association of hemophilia B and mild factor VII deficiency should be kept in mind as a peculiar form of factor IX deficiency

**Factor X variants.** The surely proven variants of factor X deficiency known today are 3 namely Mr Stuart, Miss Prower and Mrs. Minin (factor X Friuli). The main features of these 3 index patients are summarized in table I. As a matter of fact only the factor X Friuli deficiency satisfies the abnormal activation requirement proposed in the Editorial.

In Miss Prower's deficiency there is no difference between RVV-cephalin and tissue thromboplastin activation of factor X. The patient described by Denson *et al.* [1] as D.E.C. is a factor X Friuli patient who was extensively reported by us [2]. A comment in this regard was already included in a previous paper [3]. The other cases reported by Denson *et al.* [1] represent cases of classical factor X deficiency (cases M.M., G.S., L.S.) or of factor X reduced (case R.E.D.). The latter could represent a 4th factor X variant.

The patient presented by Parkin *et al.* [9] in 1974 has received no confirmation and some doubts are justified since no spe-

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## Book Reviews

*Thomas F. Necheles (ed.)*  
*The Acute Leukemias*  
Clinical Monographs in Hematology  
Thieme, Stuttgart 1979  
200 pp. 38 fig., 13 tab. DM 54.-  
ISBN 3-13-568401-6

This monograph has been written primarily for nonspecialized health care personnel and 'is meant to provide broad guidelines with sufficient references to provide a basis for further study'. The book comprises the following chapters: a short introduction on historical developments, modern classification of main types of leukemia (including appropriate light and electron microscopy figures), epidemiology and current theories about etiology is followed by comprehensive reviews about acute lymphoblastic and myeloblastic leukemias. A further chapter deals with 'unusual' forms of acute leukemia, including preleukemic states. A chapter on chemotherapeutic agents well describes features of various drugs which are in current use for induction and/or maintenance therapy. The last two chapters discuss problems of infection and future prospects in the therapy of acute leukemias, including bone marrow transplantation. References are selective and generally well chosen, covering mostly studies of the past decade (until 1978). A few typing errors e.g., 'frequently' instead of 'frequency' table 5 p. 33) are of no major consequence. The book, on the whole, fulfills the goal set by the author.

E. A. Beck, Bern

*K. W. Brunner and G. A. Nagel*  
*Internistische Krebstherapie* 2nd ed.  
Springer Berlin 1979  
X + 565 pp., 54 fig., 123 tab.,  
DM 79.-/US \$ 43.50  
ISBN 3-540-09214-5

This book has been carefully reedited, based on a first edition which was published in 1976. Its first part summarizes general concepts leading to the diagnosis and proper treatment of various malignomas. In the second part, describing specific groups of tumors and leukemias, the chapters on acute leukemias in adults, malignant lymphomas, testicular tumors and bronchogenic carcinomas have been completely rewritten, thus reflecting recent developments of therapy. Although the book emphasizes the so-called 'intensive' approach, combination therapy including surgery and radiotherapy is being discussed in a separate chapter. The personal experience of the various authors has been carefully balanced against international experience which is adequately reflected by selected citations. Tables summarizing the commercial and brand names of hormones and chemotherapeutic agents are most helpful. The subject index includes symptoms, various malignomas, complications, as well as references to drugs. The book has been reproduced from a typescript. The general presentation is nevertheless excellent, including that of illustrations and tables. This clinically oriented monograph may be recommended without restriction to every physician faced with the treatment of neoplastic diseases.

E. A. Beck, Bern

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Compiled by Barbara Boser  
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